HOST DEFENCES AGAINST *ASPERGILLUS FUMIGATUS*

Maura D Robertson

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ABSTRACT

The potential of the filamentous fungus Aspergillus fumigatus to act as an opportunistic pathogen may be related to its ability to resist the host defence network. Whilst phagocytic cells are clearly important in host defences against invading microorganisms their precise role in the killing of A. fumigatus remains undefined.

The purpose of this study was to examine the basic interactions between phagocytic cells, from humans and rodents, with spores of A. fumigatus. In particular the mechanisms whereby phagocytic cells bind and kill spores of A. fumigatus, when compared with the relatively non-pathogenic fungus Penicillium ochrochloron were investigated.

In order to investigate why people with asthma may develop some hypersensitivity reactions to A. fumigatus, in particular, rather than to the many other fungi in the atmosphere, the possibility that there may be a defect in the handling of the fungus by such patients has been tested. A comparison of the fungal handling by phagocytes from asthmatic patients, both sensitised and unsensitised to A. fumigatus with phagocytes from non-asthmatic subjects has been made.

The principal findings from this study are that spores of A. fumigatus bind to the surface of the phagocytic cell yet are relatively resistant to phagocytosis. The spores also fail to trigger the phagocytic cells into releasing the potentially

microbicidal reactive oxygen intermediates. These results may be related to a further finding which is that spores of A. fumigatus release a low molecular weight substance (diffusate) which interferes with various aspects of phagocytic cell activation. Spore diffusates were shown to inhibit the phagocytosis of radiolabelled antibody-coated sheep red blood cells and to suppress the spontaneous release of reactive oxygen intermediates by Corynebacterium parvum stimulated mouse peritoneal exudate cells. In addition spores diffusates inhibited the ability of phagocytic cells to spread on glass and reduce the number of phagocytic cells migrating towards a known chemoattractant.

Studies on spore killing showed that spores of A. fumigatus opsonised in autologous serum were more resistant to killing by phagocytic cells from humans and rodents than similarly opsonised spores of P. ochrochloron. However, the ability of the phagocytic cells to kill spores of A. fumigatus was substantially increased when the spores were opsonised in sera which had been heat-treated for 30 minutes at 56^{o}C. No increased killing was found with P. ochrochloron.

People with asthma sensitised to A. fumigatus showed significant differences in their handling of A. fumigatus in vitro when compared with the control group. Monocytes from these sensitised patients killed significantly fewer spores of A. fumigatus (opsonised in autologous sera) whilst their polymorphonuclear leucocytes killed significantly more. No such differences were found for P. ochrochloron.

The work reported in this Thesis has given us a clearer understanding of why Aspergillus fumigatus is an important cause of disease in man, and how the defence mechanisms that it has evolved in its natural environment the soil, enable it to act as a saprophyte or parasite in the lungs of humans and animals. The results also suggest a mechanism whereby heat-labile serum components may be an advantage to the survival of the fungus, thus perhaps explaining why it may be a particular problem in the airways of asthmatic patients.

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I, therefore, accept responsibility for any errors in and/or omissions from, the holograph.

MAURA D ROBERTSON

1. INTRODUCTION

1.1 The Genus Aspergillus

Aspergillus fumigatus belongs to the genus *Aspergillus*, the name given to this group of fungi by Micheli in 1729[1]. Micheli noticed a resemblance between the shape of the *Aspergillus* conidiophore and that of the aspergillum - a device used to sprinkle holy water over the congregation at church services (Fig 1.1). The genus *Aspergillus*, which belongs to the class of 'Fungi imperfecti' as it reproduces asexually by means of spores (conidia), comprises essentially saprophytic organisms which may be isolated from soil, water and decaying vegetation throughout the world[2,3]. Spores of *Aspergillus* have been recovered from places as diverse as the upper atmosphere, snow in the Antarctic and the sand and winds over the Sahara desert[2,3]. Their ubiquity and capacity to grow on many different substrates under such a wide range of environmental conditions are obviously extremely important factors in their potential pathogenicity.

The earliest account of *Aspergillus* causing disease was written at the beginning of the 19th century by Mayer and Emmert who described a case of aspergillosis in the lungs of a jay (*Corvus glandularius*)[4]. The first reports of aspergillosis in humans are usually attributed to Bennett in 1844[5] and Sluyter in 1847[6]. Bennett noted that the sputa of a man contained:

'The most beautiful and regular vegetable structures'

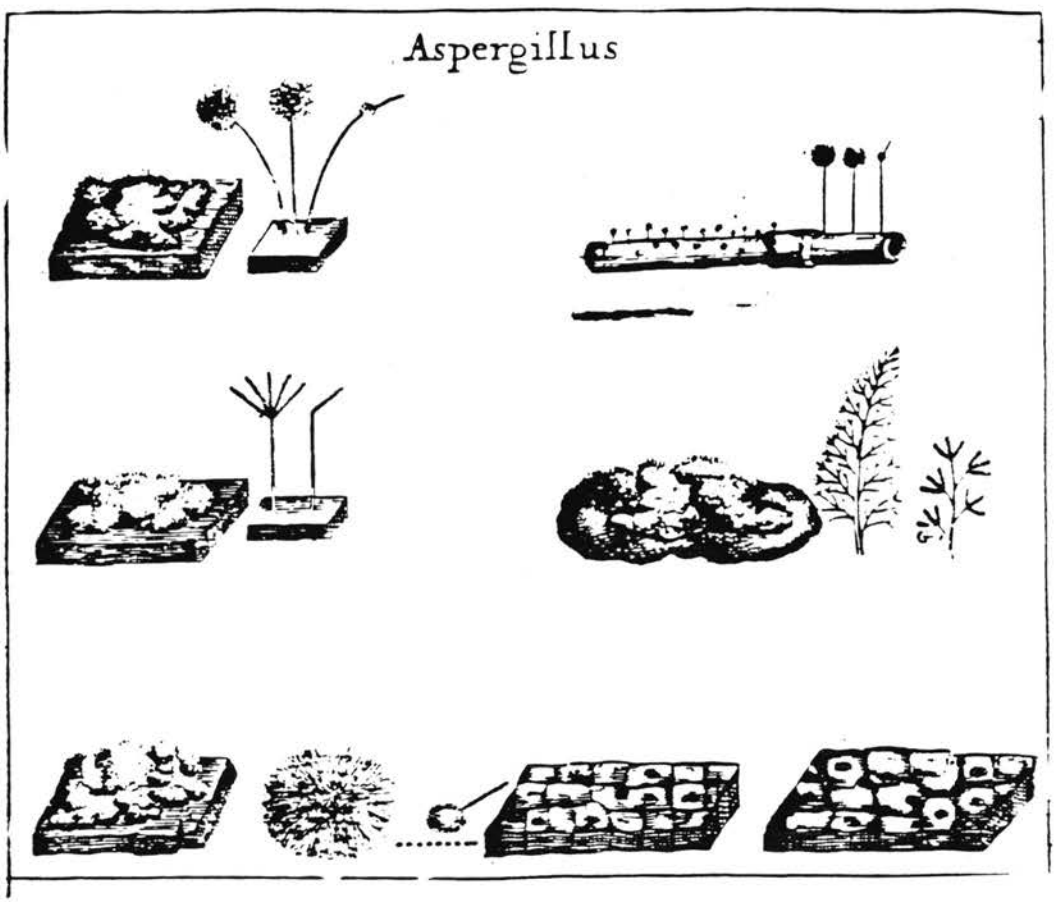


Figure 1.1 A copy of the original illustrations made by Micheli in 1729 of the genus Aspergillus[1]

Bennett also found similar vegetable matter in the tuberculous cavities of the lungs of the man at post mortem. In 1856, Virchow published a report on bronchial and pulmonary fungal disease in four patients with such an accurate description of the fungus as to allow it to be recognisable, a few years later, as A. fumigatus[7].

The name Aspergillus fumigatus (Fresen) was given to this fungus in 1863 by Fresenius who described the fungus in samples of human lung (given to him by Virchow) and in the air sac of the great bustard (Otis tarda)[8]. The association between A. fumigatus and pulmonary disease was again noted in 1897 in people employed as pigeon fatteners[9]. These people filled their mouths with a mixture of water and grain, put them over the beaks of the pigeons and transferred the mixture by breathing out. Also in 1897, Rénon published a comprehensive review of the field of aspergillosis in man and animals in which he concluded that the source of Aspergillus in the disease of pigeon fatteners was mouldy grain[10].

In 1926 Thom and Church brought all the materials on the genus Aspergillus together in a taxonomic monograph 'Manual of the Aspergilli'[11]. This was updated by Thom and Raper in 1945[12] and again in 1965 by Raper and Fennell in 'The Genus Aspergillus'[13]. Out of the 115 recognised species and varieties of aspergilli described by Raper and Fennell[13] the ones which are the most pathogenic to man and animals are the ones most frequently found in the air and derived primarily from decaying vegetation, hay, straw or grain, viz. A. fumigatus, A. flavus, A. nidulans, A. niger, A. terreus A. glaucus and A. clavatus[14]. However, out of all these species of aspergilli,

A. fumigatus is the one most commonly associated with disease in both man and animals[13] and is, therefore, the subject of this Thesis.

1.2 Growth and Dispersal of A. fumigatus

Spores of A. fumigatus are spherical and are generally 2.5-3 μm in diameter. Spores are the reproductive form of the fungi which are resistant to adverse physical conditions and preserve the species through periods when the climate is unsuitable for successful germination. However, under favourable conditions the spore will absorb fluid (usually water) and a germ tube (hypha) will emerge. The hypha elongates to form phialophores with the subsequent production of the flask-shaped conidiophore from which the unicellular spores (or conidia) are generated in chains so completing the life cycle (Fig 1.2)[13,15]. The spores are chiefly liberated by bending and breaking of spore-covered stems and leaves and during the handling or eating of hay or straw. Their aerodynamic properties aid their dispersal throughout the environment[16,17]. Although the natural habitat of A. fumigatus is the soil, using decaying organic matter as a source of food, their ability to grow over a temperature range from below 20°C up to 50°C enables them to adapt to many different situations[13]. High levels of A. fumigatus are found in decomposing vegetable matter such as self-heating hay and compost where there is a plentiful supply of food and heat[13,18,19]. An estimation of the airborne spores in a cow shed during the feeding of animals with hay and straw showed that the concentration of spores of Aspergillus vastly increased during feeding[20]. Gregory and Lacey found that large numbers of Aspergillus spores were released during the tumbling of hay[21]. Emmons in 1960

Figure 1.2 Sporing heads of A. fumigatus suspended in lactophenol-cotton blue

found that compost made of chopped leaves and branches, during the course of microbiological self-heating, became a massive and virtually pure culture of A. fumigatus; he described this work in an article entitled 'the Jekyll-Hydes of mycology'[22].

There is a seasonal variation in the incidence of spores of A. fumigatus in the atmosphere which tends to correlate with the presence of decaying organic matter. Generally spore counts of A. fumigatus are low, accounting for approximately 6% of the total fungi present in the air spora, with increased levels occurring during the winter months[17,19,23-26].

A study by Mullins et al on the release of A. fumigatus from compost heaps recorded counts of up to 170,000 spores per gramme of compost in the centre of a heap. Localised release of spores within one metre gave an average air count of 33 spores/m^3 rising to 200/m^3 in early spring whilst measurements taken 31 m away remained consistently low (33/m^3). Spore counts, in the immediate vicinity, rose to 527/m^3 after the compost was turned over once by a fork[19]. Apart, therefore, from exposure to spores in the general environment, people working in agriculture and horticulture may be exposed to higher concentrations in these localised environments.

1.3 Pathogenicity of A. fumigatus

Aspergillus illustrates particularly well the importance of the two factors in pathogenicity, the inherent properties of the organism and

the defences of the individual host. Spores of A. fumigatus act as primary pathogens in many animals, particularly those that feed on hay, straw and grain. The type of reaction occurring depends to a large extent on the susceptibility of the host and the prevalence of spores in the atmosphere. High levels of air-borne spores can cause acute respiratory distress in a number of animals[27]. Heavy losses have been reported in various species of birds including newly hatched chicks where the disease is known as 'brooder's pneumonia'[2,27-29]. Penguins in captivity seem to be particularly vulnerable to Aspergillus infections[30]. Respiratory disease by Aspergillus has also been found in young lambs[31] and piglets[14] and can cause mycotic abortion in cattle[32]. Experimental studies have shown that high concentrations of A. fumigatus in mouldy straw may cause the death of adult poultry within 24 hours[33]. Austwick in 1962 conducted a survey of the frequency of lesions containing A. fumigatus in the lungs of healthy dairy cows at slaughter and 36/49 of the animals examined showed evidence of A. fumigatus-related lesions[34].

Spores of A. fumigatus rarely act as primary pathogens in man. Instead, they have the unusual ability to manifest themselves in a variety of ways, especially in people with underlying disease[35]. In common with many other fungi, spores of A. fumigatus can be allergenic[17] provoking exacerbations of asthma especially during high seasonal concentrations of mould sporulation[36,37,38]. Some of these asthmatic patients go on to develop a hypersensitivity reaction, allergic bronchopulmonary aspergillosis[39] while non-asthmatic patients exposed to very high levels of spores may develop typical allergic alveolitis[40]. Spores may also grow saprophytically within old

pulmonary cavities to form an aspergilloma[41]. Finally, the spore may act as a true opportunistic pathogen taking advantage of the presence of local lung disease, such as infarct, tumour, pneumonia or of a generalised impairment of the host defence network related to leukopenia, leukocyte dysfunction or immunosuppressive therapy, to become either localised invasive or disseminated disease[42].

(i) Allergy: It is important to recognise that there is a spectrum of allergic responses to the antigenic components of A. fumigatus. These range from simple skin sensitisation in atopic individuals to sensitisation with asthmatic symptoms, to asthma plus occasional episodes of aspergillosis, to persistent aspergillosis with progressive lung damage[43].

Atopic subjects exposed to A. fumigatus can develop typical Type I hypersensitivity reactions[35,44] which can be measured using the skin prick test. This involves lightly scratching the skin through a solution of allergen; a positive reaction results in a wheal and flare forming, usually within 15-20 min[45]. This dermal hyper-reactivity is caused by antigenic components of A. fumigatus[46,47] binding to IgE molecules which attach via specific IgE receptors to mast cells and basophils resulting in the release of mediators and vasoactive amines and leading to increased vascular permeability, oedema and smooth muscle contraction[48,49]. Although the principal type of antibody in Type I reactions is IgE, IgG (possibly IgG_4 subclass) antibodies are sometimes present[50,51].

Allergic bronchopulmonary aspergillosis usually occurs in asthmatic individuals, although the first clinical sign of asthma may be an episode of aspergillosis[43]. It may also occur occasionally in the absence of clinical evidence of asthma, in otherwise healthy people, though most of these will prove to be atopic on skin testing[52]. Patients with cystic fibrosis are also at risk of developing bronchial colonisation by Aspergillus and may show evidence of bronchopulmonary aspergillosis[53,54].

Allergic bronchopulmonary aspergillosis was first described in 1952 by Hinson et al[39]. It characteristically affects patients with chronic asthma, who develop peripheral blood and pulmonary eosinophilia, recurring pulmonary shadowing and expectoration of golden brown sputum plugs containing hyphae of A. fumigatus. Delayed skin test reactions to intradermal injection of antigen are a common feature. In these patients, inhaled spores of A. fumigatus having deposited in the bronchi, germinate and the growth of hyphae, together with an intense immunological reaction to its presence, results in blockage of the airways. However, in most cases no invasion of the bronchial wall occurs[55,56]. The patient exhibits Type I hypersensitivity with elevated levels of specific IgE[57] and produces precipitating IgG and IgM antibodies which form immune complexes with A. fumigatus antigens resulting in Type III hypersensitivity; which occurs when antigen-antibody complexes in ratios of antigen excess are not immediately phagocytosed and disposed off. These immune complexes may, therefore, initiate the activation of complement via the classical pathway at the site of their deposition which results in the recruitment of polymorphonuclear leucocytes (PMN) which release tissue damaging

proteolytic enzymes. In addition the macrophage may become activated to secrete substances which may cause further tissue damage[58-61]. This reaction would account for the pulmonary infiltrates and damage to bronchial walls and delayed skin reactions which are not found in patients who only exhibit Type I reactions[36,53-54].

Inhalation of large numbers of air-borne conidia by both atopic and non-atopic subjects can result in the development of hypersensitivity pneumonitis (extrinsic allergic alveolitis)[62]. The development of this type of allergy following exposure to high levels of A. fumigatus, although not very common, has been found in people working with mouldy hay (farmer's lung) or compost heaps[63]. The effect of the persistent antigenic challenge is to stimulate the production of IgG precipitating antibodies in the serum which then form immune complexes with the antigen; therefore, patients usually exhibit Type III reactions. The ability of certain micro-organisms to activate complement has also been implicated in extrinsic allergic alveolitis[64,65]. Continued presence of immune complexes can result in a T lymphocyte mediated response (Type IV hypersensitivity)[44,66]. Sentitised T lymphocytes can secrete lymphokines which have a direct effect on cells involved in inflammatory responses resulting sometimes in granuloma formation[67,68]. Type IV hypersensitivity is also well recognised in patients with bronchopulmonary aspergillosis[69].

(ii) Saprophytic existence: Spores of A. fumigatus may germinate in cavitated lung tissue following damage by other disease processes, for example, tuberculosis, sarcoidosis, bronchogenic cysts, bronchiectasis, radiation fibrosis and lung abscess[70,74]. The

fungus grows within the cavity (usually preformed) to form a compact mass of mycelium called a mycetoma or aspergilloma[75]. Such lesions occupy the cavity and tend not to grow further except when they form (as happens occasionally) in the course of cavitating invasive aspergillosis, infarction or pneumonia[43,46]. Out of the 51 cases of mycetoma studied by Orie et al all were caused by A. fumigatus whilst 44 cases had pre-existing lung disease[41].

Patients with aspergilloma, unless immunosuppressed have high levels of IgG precipitating antibodies, often giving six or more arcs on immunodiffusion tests[40,58,77]. Aspergilloma nearly always occurs in non-atopic individuals although some people go on to develop allergic bronchopulmonary aspergillosis with Types I and III hypersensitivity[78]. Sepulveda found weakly positive skin test reactions to extracts of A. fumigatus in approximately 20% of the aspergilloma patients tested. Some of these patients also had A. fumigatus-specific IgE antibodies present[79]. In another study, Bardana et al found raised IgG and IgA levels in patients with aspergilloma. They did not find elevated IgE levels[80].

(iii) Opportunistic Pathogen: Aspergillus fumigatus is one of the commonest opportunistic pathogens found in the lungs of immunosuppressed patients[42,81,82]. Although the primary route of infection is through inhalation, the fungus can be locally invasive or can disseminate throughout the body resulting in intracranial, hepatic, renal, bone, thyroid and cardiac involvement[83-85]. Patients on high dose corticosteroids and those with either defective granulocyte

function or neutropenia are particularly at risk from invasive aspergillosis[86,87]. It therefore, tends to occur in people who have malignant haematological conditions, carcinoma, or chronic granulomatous disease and in those who have undergone organ transplantation[88-91]. It may occasionally occur in a wide variety of other clinical conditions, for example, following influenza or pulmonary infarction, and in the acquired immunodeficiency syndrome[92-95]. People on high doses of corticosteroids, for the treatment of chronic airflow obstruction have been known to develop invasive aspergillosis[96]. Invasive aspergillosis has also developed following resection of aspergilloma[97]. Aspergillar endocarditis with multiple systemic embolism has been described as a complication of open heart surgery due to fungal contamination of the operating theatre ventilating systems[98-100].

Invasive aspergillosis is often first diagnosed on the evidence of radiological changes coupled with seeing hyphae on sputum microscopy and culture of A. fumigatus from the sputum although the sputum may be negative[101]. At a later stage in the disease serum antibodies are sometimes present[102]. It is important that the disease is diagnosed early so that treatment can be started, as the prognosis of patients with invasive aspergillosis, especially in those with acute leukaemia is usually very poor[42,43,88,89,103].

(iv) Mixed Syndromes: The different syndromes already described (i-iii) may manifest themselves in the same patient. Locally invasive aspergillosis successfully treated may be followed by mycetoma, asthma and allergic aspergillosis converted (probably by corticosteroid

treatment) into invasive disease. Mycetoma may be complicated by asthma, and allergic aspergillosis by mycetoma[43,78,97].

The fungus A. fumigatus also produces a wide range of substances including some with mycotoxic and antibiotic properties[104-106]. Henrici in 1939 demonstrated that mycelial extracts of A. fumigatus were neurotoxic, haemolytic and able to induce histological changes in experimental animals[107]. The production of endotoxins by A. fumigatus has also been found by others[108,109]. The antibiotic substances produced by A. fumigatus include fumigatin, spinulosin, fumigacin-helvolic acid, gliotoxin and the powerful amoebicide fumagillin[110,-115]. The toxic side-effects of these substances prevent them being developed as chemotherapeutic agents in man except for fumagillin which has been used in the treatment of amoebiasis[115]. Although Poplawaska et al identified an aflatoxin-like substance in the extracts of 56 out of 109 strains of A. fumigatus isolated from 74 patients with aspergillosis its presence did not correlate with an increased frequency of unusual clinical symptoms[116]. The role that these substances might play in the pathogenesis of disease in man is unclear.

1.4 Host Defences against A. fumigatus

1.4.1 Overview of the host defence network

The mechanisms whereby higher organisms defend themselves against invading microorganisms can be separated into two categories: non-specific (innate) and specific (adaptive) immune mechanisms[117-120]. The principal components of the non-specific and specific mechanisms

(Table 1.1) act in concert to provide a highly efficient host defence system.

The lung, which is the main portal of entry of fungus into the body has a very efficient filtering system. The size, density and surface topography of the airborne spores determines their deposition among air passages and the extent of their penetration. The mechanisms used in the lung for deposition of particles larger than 1 μm are primarily sedimentation and impaction. Deposition in the large airways is predominantly due to impaction then changes to sedimentation in the small airways and alveoli as total airway cross-section increases and airway velocities drop[121]. During normal nose breathing large spores (>10 μm) become impacted in the nasal hairs which act as a mechanical barrier; however, many of these spores penetrate further and become entrapped in the mucous layer of the nasal labyrinth. They are then swept by the continually moving cilia to the pharynx and removed via the gastrointestinal tract.

During mouth breathing a higher proportion of the large spores become deposited on the ciliated epithelium of the tracheobronchial tree from where they are swept up via the mucociliary escalator towards the pharynx and swallowed. Small spores (1 μm - 5 μm) are able to penetrate the lower regions of the respiratory tract where they are deposited mainly by sedimentation. As the walls of the alveolar sacs are not ciliated the spores have to be removed by phagocytic cells (discussed in detail in section 1.4.3)[121,122]. In addition to the initial process of mechanical filtration in the lung the fluid which lines the upper and lower airways contains many substances which

TABLE 1.1 Principal Components of the Host Defence Network

NON-SPECIFIC	SPECIFIC
PHYSICAL AND MECHANICAL BARRIERS:	LYMPHOCYTES:
skin, filtering system of the upper respiratory tract, membranes lining the body tracts	B cells, T cell (lymphokines, direct action)
BODY FLUIDS:	ANTIGEN PRESENTING CELLS:
acids, lysozymes, immunoglobulin complement, acute phase proteins, surfactant, phagocytic cell products	dendritic cells, macrophages, T cells B cells
PHAGOCYTIC CELLS:	
PMN, monocytes, macrophages	

prevent microbial invasion[121]. The respiratory tract fluids constitute a physical barrier to particulate contact with bronchial and alveolar epithelia. The secretions that coat the ciliated epithelia form the viscoelastic medium in which the cilia beat, propelling particles on the mucociliary escalator. This fluid also contains many substances with antimicrobial properties including lysozyme, immunoglobulins: IgG and secretory IgA (11S IgA), complement components, lactoferrin and protease inhibitors[121-124].

These non-specific mechanisms, for example the mechanical filtration of the lung or physical barrier of the skin are used as the first lines of defence to prevent microorganisms invading the tissues. However, if the microorganism is able to breach this barrier it may be adequately dealt with by the humoral and cellular components of the body fluids, in particular, the phagocytic cells. These have the capacity to engulf the microorganism and produce an armoury of substances including enzymes and reactive oxygen intermediates which in addition to complement components have potential microbicidal properties[119,125-133]. The efficiency with which the micro-organism is eliminated by the phagocytic cells often determines whether a specific immune response is required[134,-136]. The central component of the specific defence mechanism is the lymphocyte. The T and B lymphocytes work in concert with the phagocytic cells to mount humoral and cell-mediated immune responses which are specifically directed at the offending microorganism[134-139]. A breakdown or deficiency in any one of these components of either the non-specific or specific defence mechanisms renders the individual more susceptible to opportunistic infections[140-144]. Classical examples of A. fumigatus

taking advantage of a defective host defence network are aspergillar endocarditis (A. fumigatus entering the tissue at the site of incision during surgery) and invasive aspergillosis in people with PMN dysfunction either alone (e.g. as in chronic granulomatous disease) or combined with lymphocyte dysfunction (e.g. as in acute leukaemia or in subjects receiving cytotoxic drugs)[82,88,91,98,100].

1.4.2 A. fumigatus in the lung

The normal portal of entry of A. fumigatus into the body is the lung[145]. Spores of A. fumigatus can, because of their small size (<5 um) and their aerodynamic properties, bypass the upper respiratory tract defences and may reach distal regions of the lung[17,146,147]. In this region the host defences rely upon phagocytic cells to remove the spore efficiently[148,149]. If, however, the phagocytic cells are unable to clear the spore quickly, germination and fungal colonisation may occur[150]. A possible relationship between isolation of A. fumigatus and lung disease was shown in 1959 by Pepys et al who cultured spores of A. fumigatus more frequently from the sputum of asthmatic patients than from the sputa of people with other lung diseases[151]. Mullins and Seaton who were able to show that spores of A. fumigatus were recovered from post mortem lung specimens more frequently than would have been anticipated from their prevalence in the air spora, suggested that this fungus may have a specific ability to resist the natural defences of the lung[152].

Experimental animal studies have also shown that spores of A. fumigatus appear to be fairly resistant to the normal host defences of the lung[153-160]. Treatment of animals with corticosteroids prior

to challenge with spores of A. fumigatus, substantially impairs their host defences and renders them highly susceptible to disseminated infection[153,154]. Sandhu et al in 1970 exposed mice by inhalation, to seven different species of Aspergillus (A. fumigatus, A. flavus, A. tamarii, A. nidulans, A. nidulans var dentatus, A. niger, A. terreus) and found that corticosteroid treatment lowered the host resistance to all seven species. However, A. fumigatus was the most pathogenic causing the highest mortality in the corticosteroid group (13 out of 15) as well as in the untreated group (4 out of 15)[153]. This effect of corticosteroid treatment was also shown by White in 1977, who found that inhaled conidia of A. fumigatus germinated at a high rate in the lungs of cortisone-treated mice when compared with naive mice[154]. A study on the pathogenesis of experimental pulmonary aspergillosis in normal and cortisone-treated rats by Turner et al in 1976 showed that rats were resistant to infection following intratracheally administered spores of A. fumigatus even although they developed a subacute interstitial pneumonia. Although the lesions were more severe in animals whose inflammation was suppressed with corticosteroids there was no evidence of hyphal growth in the pulmonary tissue. They postulated that phagocytosis, followed by digestion of spores, was of paramount importance in the control of fungal infections[155].

Rabbits exposed to spores of A. fumigatus and Penicillium sp. which were inhaled or instilled directly into the stomach, did not produce precipitating antibodies, even though fungi were isolated from the tissue 16 days after exposure[157]. Experimental studies of the specific host defences against A. fumigatus by Smith, Williams et al,

and by Lehmann and White (1976) showed that mice, which had been pre-treated with a sublethal dose of A. fumigatus spores were more resistant to a subsequent challenge with a higher dose[158,159,160]. Eskenasy and Molan immunised rabbits with antigens of Aspergillus in order to produce an animal model with a high titre of specific antibodies to A. fumigatus. They challenged these sensitised and non-sensitised rabbits intratracheally with spores of A. fumigatus and compared the histopathological reaction. The lung tissue of rabbit sensitised with aspergillar antigens showed extremely active granulocytic and macrophage alveolar reactions whilst the titre of serum antibody rapidly dropped; all animals survived. In contrast the unsensitised animals developed a rapid exudative-necrotic alveolar reaction with a high mortality[161].

The importance of the specific immune response in the elimination of A. fumigatus nevertheless remains unclear. Turner et al, using rats showed that antibodies to A. fumigatus did not exert a protective role against the development of fungal infections and may even have accelerated the disease[162]. Williams et al showed that mice with impaired T cell function could still resist infection with A. fumigatus; however, they were not as efficient at dealing with a second challenge as were mice who were T cell competent[159].
Taken together all these findings have led to the suggestion that prevention of infection with A. fumigatus is primarily dependent on the action of the phagocytic cells. Therefore examination of the role that phagocytic cells may play in the eradication of spores of A. fumigatus is the central theme of this Thesis.

1.4.3. Phagocytic cells

The importance of the phagocyte in the eradication of microorganisms was first described by Metchnikoff over 100 years ago[163]. Metchnikoff observed that cells which he named 'microphages' and 'macrophages' struggle against a wide variety of foreign bodies and living organisms by attempting to ingest and degrade them intracellularly. He understood these cellular actions to be the basis of an organism's inflammatory response to wounding[163]. We now know that microphages (polymorphonuclear granulocytes) and macrophages (mononuclear phagocytes) originate in the bone marrow from a common stem cell, differentiate along separate pathways, before circulation in the blood[164,165]. The granulocytes which comprise primary PMN, as well as eosinophils, basophils and tissue mast cells are short-lived cells with a half life of only a few days. When required PMN are rapidly mobilised into the tissue to sites of inflammation[166,167]. Monocytes remain in the circulation for a few days and then migrate to the tissues to become long-lived tissue macrophages[167,168]. Other cells have been shown to exhibit limited phagocytic ability; these include endothelial cells, epithelial cells and fibroblasts, referred to as facultative or 'non-professional' phagocytes[169,170]. PMN and mononuclear phagocytes are the 'professional' phagocytes because they are rapidly mobilised to infectious foci and can phagocytose and kill microorganisms[170].

Phylogenetically phagocytosis is the most established defence mechanism against foreign invaders. Among primitive forms of animal life (for example, the amoebae), phagocytosis is the principal means of ingesting food. This application of phagocytosis antedates its

involvement into a defence mechanism among the more advanced multicellular animals[171]. The process of phagocytosis can for ease of description, be arbritarily divided into four phases - (i) recognition and chemotaxis, (ii) attachment, (iii) ingestion and (iv) digestion.

(i) Recognition and chemotaxis

Phagocytic cells have the capacity to discriminate between foreign and self materials as well as alterations to self. When phagocytic cells first encounter foreign microorganisms, whether by chance or by specific attraction to the cell surface of the micro-organism, they are able to secrete or initiate the production of mediators which can mobilise other phagocytic cells to the site of inflammation[172]. The ability of cells to migrate unidirectionally in response to chemical signals enables them to accumulate at the site of inflammation. Three types of cellular motility can be defined, (i) random migration (ii) chemokinesis-stimulated random migration due to substances that enhance phagocyte locomotion, (iii) chemotaxis - unidirectional locomotion of cells along a concentration gradient of a chemoattractant: this is the principal mechanism[173]. Many substances attract or are positively chemotactic for phagocytes (Table 1.2)[174]. One of the most potent chemotactic factors generated during phagocyte activation is the complement component C5a[175]. Many microorganisms (including A. fumigatus) have been shown to directly stimulate the production of chemotactic factors from the complement system as well as the fibrinolytic and kinin generating systems[176-178].

Recognition (as well as attachment) of microorganisms by phagocytosis

TABLE 1.2 Some of the factors found to be chemotactic for phagocytic cells

Complement derived

C5a ($C5a_{desarg}$), C3bBb

Cell derived

Leukotriene B_4, 5-HETE
Neutrophil release products induced by urate crystals, aggregated IgG, phagocytosis
Macrophage release products
Lymphokines
Mast cell release products - ECF-A

Miscellaneous

Products of tissue damage
Caseins (α_s - and β-casein)
Synthetic formyl-methionyl peptides
Bacterial signal peptides, bacterial lipids
Tumour cell supernatants
Pulmonary surfactant lipoprotein
Products of the fibrinolytic and kinin-generating systems

is also promoted by coating them with serum factors called opsonins which were first described by Wright and Douglas in 1904[179]. The two principal serum opsonins are immunoglobulins (mainly IgG_1 and IgG_3) and complement (C3b, C3d)[180, 181] but other serum proteins are thought to have opsonic activity. These include alpha and beta globulins and C- reactive protein (reviewed by Stossell, 1975)[182].

(ii) Attachment

The attachment process involves the apposition of phagocytic cell membrane and the surface of the microorganism. Attachment can occur in the absence of cellular metabolism and is dependent upon the interplay of surface forces (surface tension, surface-free energy) cell surface receptors, structural properties of the plasma membrane, as well as the molecular nature of the microorganism[126,183,184]. Surface receptors present on phagocytic cells include those for complement (CR1, CR2, CR3) immunoglobulins (FcRI, FcRII), lectin/sugars, lymphokines and hormones[184,185,186]. The most extensively studied of these receptors are those for the principal opsonins, complement and immunoglobulin. Specific antibody binds to the microorganism via its $(Fab')_2$ region; the antibody coated microorganism may then bind to the phagocytic cell via the Fc receptors on the cell surface[187]. Interaction of specific IgM antibodies with the microorganism leads to activation of the complement pathway; this results in the deposition of C3 components (mainly C3b) on the microorganism[188]. The C3b coated microorganism then binds to the phagocytic cells via complement receptors[189]. Microorganisms which have been found to activate complement directly via the alternate pathway may become coated with C3b which could result in their attachment to the phagocytic cell in

the absence of specific antibody[64,65,190-192].

Other receptors which bind the complement component iC3b are MAC-1, LFA-1 and p150,95; these receptors have been shown to be closely related to the CR_3 receptor[193-195].

The ability of phagocytes to adhere to various surfaces in the absence of antibody or complement is a well-known phenomenon[196,197]. Lectin-like receptors which can recognise carbohydrates on the surfaces of microorganisms are thought to be part of a more primitive non-specific system of recognition[198,199]. These receptors in the mouse appear to be closely associated or identical to Ia antigens of the major histo-compatability complex[200].

Perturbation of the plasma membrane may induce a phagocytic cell to undergo a respiratory burst with the production of substantial quantities of superoxide anion and hydrogen peroxide. These products may interact to form a variety of short-lived oxygen species (for example, hydroxyl radical, singlet oxygen) and longer-lived oxidants (for example hypochlorous acid, monochloramine). These reactive oxygen intermediates constitute key components of the oxygen-dependent antimicrobial and cytocidal mechanisms of phagocytic cells[201-203]. In addition to the reactive oxygen intermediates membrane perturbation also induces the phagocytic cell to secrete a vast array of substances including enzymes and chemotactic factors which are involved in the eradication of the microorganism (see Table 1.3)[204-206].

TABLE 1.3 Some of the secretory products produced by PMN and/or mononuclear phagocytes implicated in anti-microbial defense

REACTIVE OXYGEN INTERMEDIATES:

Superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radical

COMPLEMENT COMPONENTS:

C_1, C_4, C_2, C_3, C_5, factor B, factor D, properdin C1b inactivator, B-IH

OTHER ENZYMES:

Neutral proteases, elastase, lysosyme, lysosomal acid hydrolases, catalase, myeloperoxidase

HORMONES:

Neutrophil activating factor, interleukin-1, interferon, cachectin, colony stimulating factor for granulocytes and macrophages, thyroid hormones

OTHERS:

Platelet activating factor, lipoxygenase and cyclo-oxygenase products, chloride, lactic acid, lactoferrin, Granular cationic proteins transferrin

(iii) Ingestion

Attachment of microorganisms via specific receptors can trigger the cell to undergo the process of ingestion which is highly complex and involves a coordinated interaction of the plasma membrane with contractile elements in the cytoplasm. Phagocytes surround objects with an area of peripheral cytoplasm which excludes organelles (called hyaline-ectoplasm). The pseudopodia of the phagocytic cell extend around the microorganism until the membrane fuses to form the phagosome[207,208]. Since the cell actively crawls around large objects, the machinery involved in locomotion and in phagocytosis of large objects is nearly identical. The motor elements for phagocytosis are mediated by an interaction of actin with other proteins and the system is usually controlled by ionised calcium. Actin filaments interact with myosin in the presence of adenosine triphosphate to cause contraction of the dispersed filaments into aggregates. Each contractile unit is a set of actin filaments linked by myosin filaments engaged in a 'tug of war' for the actin filaments. The net result is propogation of the pseudopodia and ingestion of the object[208].

During ingestion of microorganisms phagocytic cells release many antimicrobial substances including reactive oxygen intermediates and lysosomal enzymes[204,205,206,209].

(IV) Digestion

During and after formation of the phagosome, adjoining lysosomes fuse with the phagocytic vacuole and the contents of the lysosome discharge

into the phagosome to form a phagolysosome[210]. The lethal combination of oxygen metabolites, radicals, ions and enzymes ensures the rapid destruction of most microorganisms within this struture[211].

1.4.4 Mechanisms of resistance to phagocytic cells

A number of microorganisms have developed mechanisms of resistance to the phagocytic processes[212]. Adult schistosomes are able to acquire red cell determinants and MHC Class I antigens of the host on their surface; these could mask the foreign antigens of the schistosomes, thereby enabling them to evade recognition by the eosinophil of the adult worm[213,214]. Many strains of pathogenic bacteria, eg Staphylococcus aureus and Escherechia coli have surface structures (capsules) that inhibit their binding and ingestion by phagocytic cells[215]. Many pathogens are not readily ingested by phagocytic cells because of antiphagocytic substances on their surfaces, for example Candida albicans hyphae and slime-producing strains of Pseudomonas aeruginosa[216,217]. For intracellular pathogens to survive they must have developed means to circumvent the microbicidal actions of the granules which empty into the phagosome during phagolysomal fusion as well as those produced by the phagocyte during ingestion. The ability of Toxoplasma gondii to survive within certain mononuclear phagocytes is dependent in part upon it becoming phagocytosed without eliciting the production of reactive oxygen intermediates[218]. The cell wall of Mycobacterium tuberculosis contains a substance which inhibits degranulation of the lysosome. However, M. tuberculosis and many other organisms can survive within phagolysosomes[219,220]. A more comprehensive list of microorganisms which have been shown to have mechanisms of resistance is given in

Table 1.4 (adapted from the review by Densen and Mandell).

In comparison to the vast amount of literature on the importance of phagocytic cells in the eradication of bacteria and viruses, little is known about their role in the host defences against fungal infections. However, it is generally thought that the mechanisms used are essentially similar to those involved in the resistance to bacterial infections.

1.4.5 Phagocytic cells versus A. fumigatus

The importance of the phagocytic cell in the eradication of spores of A. fumigatus was highlighted in 1897 by Rénon who injected spores of A. fumigatus into the peritoneal cavities of guinea-pigs and rabbits; for comparison he used spores of A. niger which he considered to be non-pathogenic[10]. After three hours he examined the peritoneal exudates and found that the majority of spores of A. niger were then within the leucocytes. However, the majority of spores of A. fumigatus were still lying free within the exudate (Fig 1.3). Rénon also demonstrated that spores of A. fumigatus were still present though not germinating in the lymphatic sac of frogs 35 hours after injection (Fig 1.4). Rénon suggested that the 'poor response' of the leucocytes was the reason for pathogenicity of A. fumigatus. In the 90 years since the pioneering work of Rénon our knowledge of the role that phagocytic cells play in the eradication of A. fumigatus has not really progressed. The few published studies on the interaction of phagocytic cells with spores of A. fumigatus have produced conflicting results. Kurup found that rabbit alveolar macrophages killed 80% of spores of A. flavus, 60% of A. fumigatus and only 30% of

TABLE 1.4 Mechanisms of resistance of some microorganisms to the actions of phagocytic cells

INHIBITION OF PHAGOCYTE RECOGNITION OR CHEMOTAXIS

Salmonella typhi
Neisseria meningitidis
Neisseria gonorrhoeae
Pseudomonas aeruginosa
Serratia species
Mycobacterium tuberculosis
Staphylococcus aureus
Capnocytophaga
Escherichia coli
Vibrio cholera

INHIBITION OF ATTACHMENT TO/OR INGESTION BY PHAGOCYTE

Streptococcus pneumoniae
Streptococcus pyogenes
Klebsiella pneumoniae
Haemophilus influenzae
Yersinia pestis
Bacillus anthracis
Campylobacter fetus
Cryptococcus neoformans
Pasteurella multocida
Bacteroides fragilis
Mycoplasma
Influenza virus
N. meningitidis
N. gonorrhoea
P. auruginosa
S. aureus
E. coli
C. albicans

INHIBITION OF PHAGOCYTE RESPIRATORY BURST OR DEGRANULATION

Brucella abortus
Herpex simplex virus
Influenza virus
K. pneumoniae
M. tuberculosis
Newcastle disease virus
Toxoplasma gondii
Reovirus
Vaccinia virus

RESISTANCE TO OXIDATIVE OR ENZYMATIC ATTACK

S. typhi
B. abortus
Salmonella minnesota
E. coli
Listeria monocytogenes
Mycobacterium bovis
M. tuberculosis
Mycobacterium leprae

MICROBIAL TACTICS ESCAPE FROM PHAGOSOME

Reovirus
M. bovis
Rickettsia tsutsugumushi
Vaccinia virus

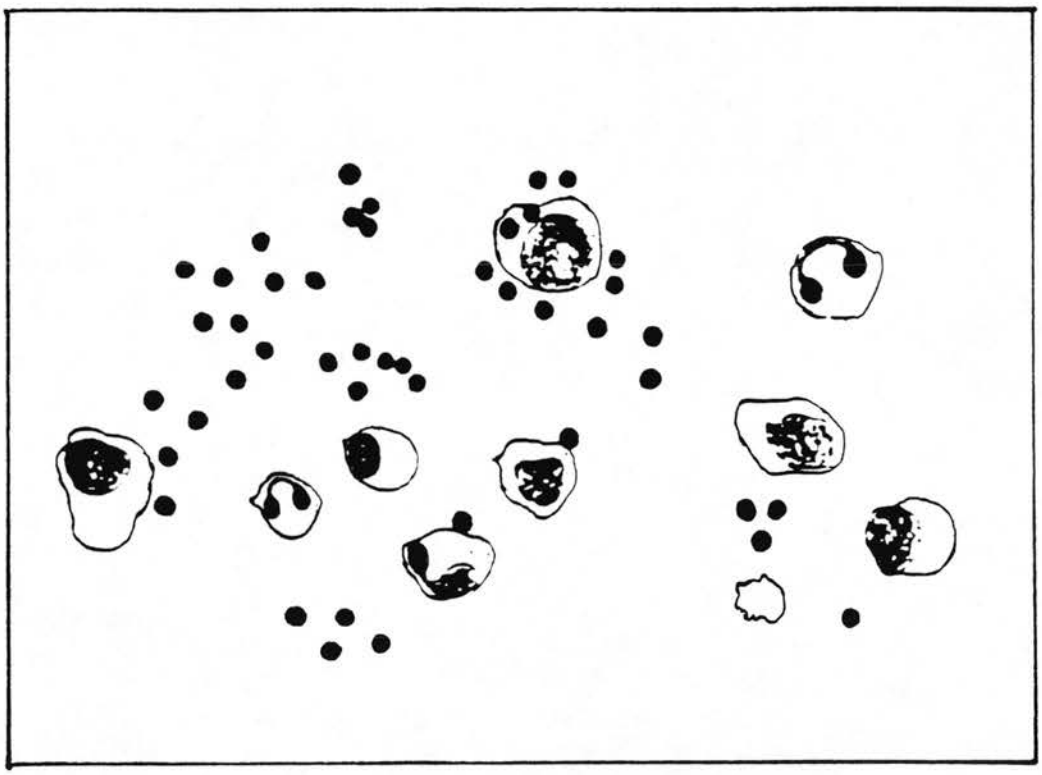


Figure 1.3 A copy of the original illustration made by Rénon in 1897 of the interaction between spores and cells, found in the peritoneal exudate which was obtained from guinea pigs, 3 h after receiving an intraperitoneal injection of spores of A. fumigatus[10]. The majority of spores (●) are lying free in the exudate.

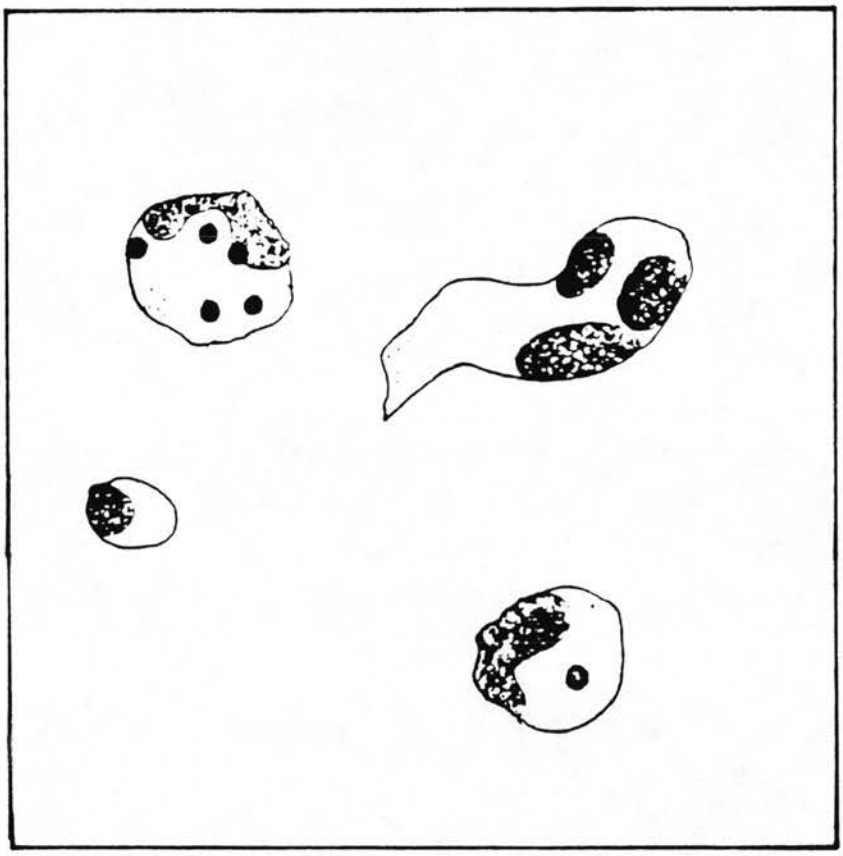


Figure 1.4 A copy of the original illustration made by Rénon in 1897 of the spores of A. fumigatus (●) contained within leucocytes taken from the lymphatic sac of a frog 35 h after inoculation[10].

A. niger following a two hour incubation in vitro. However, from these results he concluded that rabbit alveolar macrophages effectively killed all three fungi[221]. The results of another study by Schaffner et al comparing the ability of phagocytic cells, from different anatomical sites, to kill these three fungi showed that alveolar macrophages from mice and rabbits were more efficient than peritoneal macrophages in the prevention of germination of all three aspergilli. Schaffner et al found that the alveolar macrophages from rabbits killed 82-90% of spores of A. fumigatus within 30 hours whilst, in contrast, the ability of peritoneal macrophages to inhibit germination of the ingested spores was substantially less[222]. The results of another study comparing natural immunity to A. fumigatus in vivo with the action of phagocytes against the organism in vitro, led Schaffner et al to suggest that monocytes may be concerned with killing spores while PMN may be responsible for the eradication of hyphae, apparently in the absence of a specific immune response[223]. Diamond et al have also shown that PMN as well as monocytes can damage hyphae[224,225]. Lehrer and Jan found that serum was necessary for optimal phagocytosis of spores of A. fumigatus by human leucocytes and its opsonic activity was greatly diminished by heat inactivation (56^{o}C, 30 min). However, even after incubation for three hours with human monocytes and PMN the phagocytosed spores remained viable[226]. In a later study by Kurup, in vitro experiments showed that pulmonary alveolar macrophages from normal rabbits were relatively ineffective at killing spores of A. fumigatus in four hours when compared with the high percentage of spores killed of A. flavus (90%) and A. niger (18.9% compared with 90% and 82% respectively). Prior opsonisation of the spores in normal rabbit serum, rabbit anti-A. fumigatus sera,

complement or lung lavage fluid had no real effect on the phagocytosis or killing of the spores[227]. Waldorf et al showed that alveolar macrophages of mice, challenged in vivo continued to kill spores of A. fumigatus when cultured in vitro[228]. The results of two case reports have shown that phagocytic cells from patients with recurrent aspergillus infections are unable to kill spores of A. fumigatus efficiently[229,230]. This led Fietta et al to suggest that oxygen-independent mechanisms could play a basic role in Aspergillus killing[230].

The potential importance of reactive oxygen intermediates and of hydrogen peroxide in particular for the eradication of spores and hyphae has been demonstrated with cell-free systems of myeloperoxidase-hydrogen peroxide-iodide[231,232] and ferrous iron-hydrogen peroxide-iodide[233] both of which are capable of killing A. fumigatus. Although Lehrar and Jan found that the myeloperoxidase-hydrogen peroxide-iodide system was efficient at killing spores of A. fumigatus in vitro they also showed that spores of A. fumigatus were much more resistant to the myeloperoxidase-hydrogen peroxide-chloride system. As chloride is much more readily available to phagocytic cells than iodide they suggested that the relative ineffectiveness of chloride in the system could underlie the apparent inability of human neutrophils to kill ingested spores of A. fumigatus[226].

It is, therefore, apparent from this review of the literature that there is still considerable doubt and even confusion about the mechanisms that may be used by phagocytic cells in the eradication of this fungus.

1.5 Aims of the Thesis

The principal aim of this work was to examine the ability of phagocytic cells to handle spores of A. fumigatus with the intention of increasing our understanding of the mechanisms involved in the pathogenesis of aspergillar diseases. The interactions between phagocytic cells, from humans and rodents, with spores of A. fumigatus were examined with particular emphasis on the different stages of the phagocytosis and killing process.

To test the hypothesis that asthmatic patients, specifically sensitised to A. fumigatus, may have a defect in their handling of this fungus, a comparison of the handling of fungi by phagocytes from asthmatic patients, both sensitised and unsensitised to A. fumigatus with that by phagocytes from non-asthmatic subjects has been made. In order to facilitate interpretation of both in vivo and in vitro studies, spores of the non-pathogenic P. ochrochloron were used as a control.

2. MATERIALS AND METHODS

A number of different methods were used to examine the interaction of phagocytic cells and fungal spores. As the majority of these methods were routinely used in the laboratory at the I.O.M. no further standardisation was required. However, some of the methods used did require standardisation, in particular, the fungal spore killing assay. The principal results of the standardisation experiments can be found in this chapter under the specific method description.

2.1 Selection of Subjects

Prior to commencement of the study approval was obtained from the local Ethics of Research Committee. Informed consent to donate blood for the research was obtained from all participants.

The subjects chosen for this study fell into three main groups:

(i) asthmatic subjects non-sensitised to *A fumigatus*,

(ii) asthmatic subjects sensitised to *A. fumigatus* (including patients with allergic bronchopulmonary aspergillosis),

(iii) non-asthmatic control subjects

The majority were patients attending the Chest Clinic at the Royal Victoria Dispensary (RVD), Edinburgh. In addition patients with allergic bronchopulmonary aspergillosis were recruited with the co-operation of the consultant chest physicians in the Edinburgh area. The control group consisted of non-asthmatic individuals, the majority of whom were members of staff at the Institute of Occupational

Medicine (IOM); some were patients attending RVD for follow-up of other conditions. A total of 58 people participated in this study. The age distribution within the different groups is given in Table 2.1.

2.2 Completion of Questionnaire

All participants in the study were asked to answer questions relating to their age, smoking habit and medications (Fig. 2.1).

2.3 Skin Testing

All subjects were skin tested by the prick-test method using standard Bencard allergy preparations (Bencard, Brentford, England). The allergens used were: A. fumigatus, Dermatophagoides pteronyssinus, grass-pollen, Penicillium sp: in addition a control solution (without added allergen) was applied. The flexor aspect of the forearm was used for testing and a ballpoint pen was used to label the patient's arm adjacent to the test sites in order to identify the allergen and control solutions. Using the applicator, one drop of each of the allergen solutions was applied to the skin and a needle was used to lightly puncture the skin below the solution; a fresh needle was used for each of the allergens. After 15 min the arm was examined for a wheal and flare reaction and the diameter of the wheal, if any, was recorded. A response greater than that found with the control was recorded as positive. All patients who gave a skin test positive reaction to A. fumigatus were included in the asthma-sensitised group.

TABLE 2.1 Age distribution of the people from each of the clinical groups

Description	CLINICAL GROUP		
	Control	Asthmatic Non-sensitised	Asthmatic Sensitised
Number of cases	20	19	19
Age Range	21-65	15-72	17-79
Mean (SD) of age	40.6 (14.4)	43.4 (19.8)	41.9 (16.7)

Fig 2.1 The questionnaire completed for each subject participating in the study

THE HOST DEFENCES AGAINST ASPERGILLUS FUMIGATUS

1. IDENTITY INFORMATION

CASE NO. (enter preceding zeros) _ _ _ _ _ _

SAMPLE NO. (enter preceding zeros) _ _ _ _ _ _

SURNAME _____

INITIALS _____

SEX (enter M = male or F = female) __

DATE OF BIRTH (enter DDMMYY with preceding zeros) _ _ _ _ _ _

DIAGNOSIS _____

_____

_____

NATURE OF SAMPLE (enter B = blood or L = lung lavage) __

DATE SAMPLE TAKEN (enter DDMMYY with preceding zeros) _ _ _ _ _ _

now go to section 2 (smoking history)

2 SMOKING HISTORY

(a) DO YOU SMOKE? (if yes enter Y and go to question (b)) ___

(if no enter N and go to question (d)) ___

(b) WHICH OF THE FOLLOWING DO YOU SMOKE REGULARLY?

CIGARETTES (if yes enter Y, if no enter N) ___

CIGARS (if yes enter Y, if no enter N) ___

PIPE (if yes enter Y, if no enter N) ___

(c) ON AVERAGE, HOW MANY DO YOU SMOKE PER WEEK?

CIGARETTES (enter preceding zeros for amount or leave blank if not applicable) _ _ _ _

CIGARS (enter preceding zeros for amount or leave blank if not applicable) _ _ _ _

OUNCES OF TOBACCO (enter preceding zeros for amount or leave blank if not applicable) _ _ _ _

for 1/4 oz. record 00.25
1/2 oz. record 00.50
3/4 oz. record 00.75 etc)

now go to section 3 (medication & skin test results)

2 SMOKING HISTORY (continued)

(d) HAVE YOU EVER SMOKED AS MUCH AS ONE CIGARETTE A DAY (OR ONE CIGAR A WEEK OR ONE OUNCE OF TOBACCO A MONTH) FOR AS LONG AS A YEAR? ___

(if yes enter Y and go to question (e))

(if no enter N and go to section 3)

(e) IS IT LESS THAN A YEAR SINCE YOU STOPPED SMOKING? ___

(if yes enter Y and go to section 3)

(if no enter N and go to question (f))

(f) HOW MANY YEARS IS IT SINCE YOU STOPPED SMOKING? _ _ _ _

(enter with preceding zero)

now go to section 3. (medication & skin tests results)

3 MEDICATION

(g) ARE YOU TAKING ANY MEDICINES? ___

(if yes enter Y and go to question (h))

(if no enter N and go to section 4)

(h) WHICH MEDICINES ARE YOU TAKING?

_____

_____

_____

_____

_____

4 SKIN TESTS RESULTS (record all results in millimetres with preceding zeros)

CONTROL ____

ASPERGILLUS FUMIGATUS ____

DERMATOPHAGOIDES PTERONYSSINUS ____

GRASS POLLENS ____

PENICLLIUM SP. ____

ADDITIONAL TESTS (please specify) ____

____

2.4 Collection of Blood

A 30 ml sample of blood was withdrawn from an antecubital vein. Twenty five millilitres of blood were transferred into a plastic universal container (Nunc, Gibco Europe Ltd, Paisley, Scotland) containing the anticoagulant ethylenediaminetetra-acetic acid, dipotassium salt (EDTA-Sigma Chemical Co. Ltd., Dorset, England) to give a final concentration of 15 mg EDTA/1 ml blood. The remaining 5 ml of blood were transferred into a sterile glass tube (New Brunswick Scientific Ltd., London) and allowed to clot at room temperature (RTP). Sterile technique were used throughout.

2.5 Separation of Mononuclear and Polymorphonuclear Cells

The cells were isolated by density gradient centrifugation using the basic method described by Ferrante and Thong[234]. The principles of this method were established by Böyum in 1968[235]. A sterile plastic Pasteur pipette (Sterilin Ltd, Middlesex, England) was used to layer gently 3.5 ml of the anticoagulated blood onto 3 ml of Mono/Poly resolving medium density 1.114 $\pm$ 0.002 (Flow Labs., Irvine, Scotland) in a plastic sterile conical centrifuge tube (Sterilin). This was centrifuged in a MSE Mistral 400 centrifuge for a total of 40 min at room temperature. For the first 30 min the blood was spun at 300 *g* followed by 10 min at 400 *g*. This technique allows cells to be separated according to their densities illustrated in Fig 2.2. The top layer which contained the mononuclear cells was removed first followed by the second layer which contained the polymorphonuclear (PMN) cells. Cell yield was increased by using a sterile plastic Pasteur pipette and a circular motion of the hand around the cell

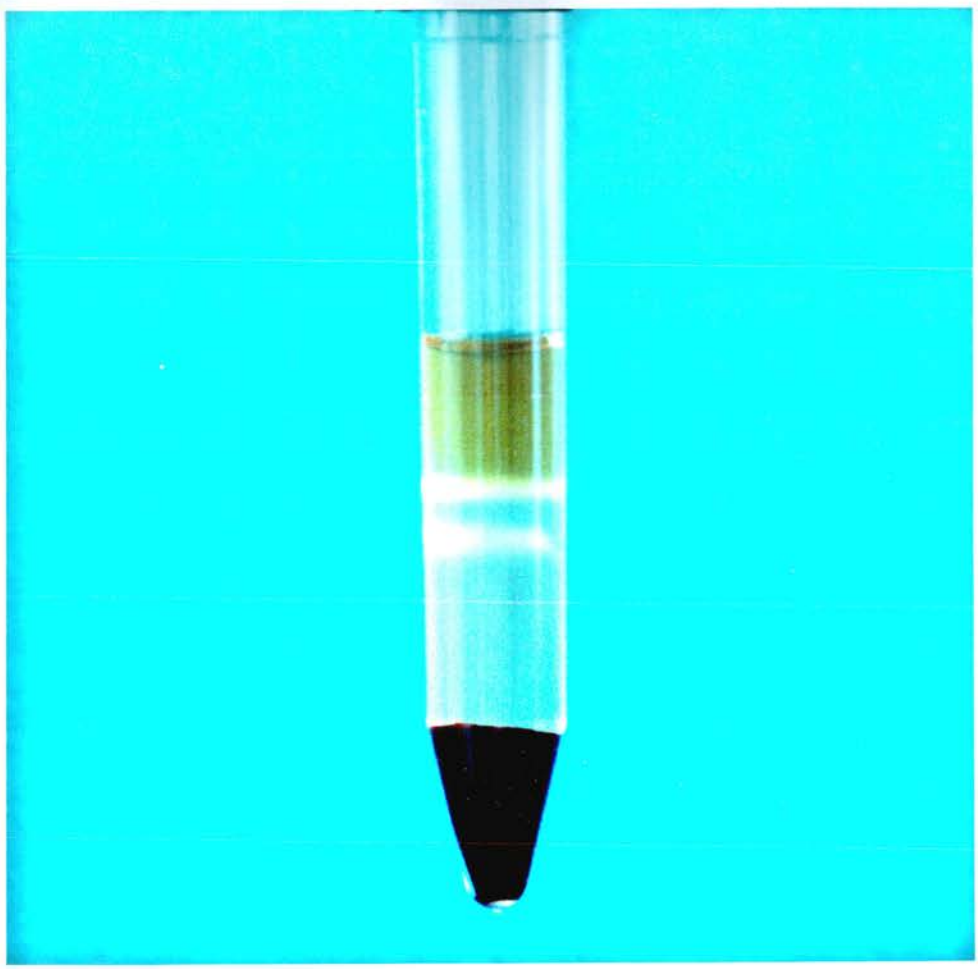


Figure 2.2 Mono/Poly resolving medium separation of human peripheral blood in a conical tube following centrifugation for 40 min. From the top down; straw-coloured layer is plasma, first white band contains the mononuclear cells, second white band contains the granulocytes. The bottom dark layer contains the red blood cells.

TABLE 2.2 The number of mononuclear cells and granulocytes isolated from 25 ml blood using Mono/Poly medium:divided by clinical group

Clinical	Number in group	Mononuclear	PMN
		Total Cells x 10^6	
Control	15	20.96 (6.2)[1]	18.14 (9.7)
Asthma non-sensitised	14	24.17 (8.6)	25.82 (12.9)
Asthma sensitised	13	22.05 (10.9)	27.2 (15.8)

1 Mean (SD)

TABLE 2.3 Differential white blood cells present in the mononuclear and granulocyte cell layers, isolated using MONO/POLY medium, from the blood of subjects divided by clinical group

Clinical Status	Number in group	Mono/Poly layer	DIFFERENTIAL % PMN	Lymphocytes	Monocytes	Eosinophil
Control	15	Mono	2.08 (1.9)[1]	58.0 (8.4)	39.77 (8.2)	0.23 (0.8)
		Poly	91.47 (6.8)	3.6 (6.5)	0.13 (0.5)	4.87 (4.2)
Asthma Non-sensitised	14	Mono	2.79 (4.5)	56.5 (10.2)	41.43 (11.26)	0.07 (0.3)
		Poly	90.0 (7.4)	1.85 (1.3)	0 (0)	8.15 (6.8)
Asthma Sensitised	13	Mono	1.54 (1.5)	47.0 (10.8)	50.23 (9.47)	0.15 (0.6)
		Poly	91.62 (5.6)	1.92 (1.6)	0 (0)	6.46 (5.1)

1 Mean (SD)

TABLE 2.4 White blood cell count of subjects divided by clinical group

Clinical Group	Number in Group	WBCC x 10^9/l Mean (SD)
Control	15	6.74 (2.01)
Asthma Non-sensitised	14	7.17 (1.55)
Asthma Sensitised	13	7.49 (1.98)

layer interface. The cell layers were transferred into ice-cold conical centrifuge tubes (Sterilin) and washed twice in phosphate buffered saline (PBS) [without calcium (Ca^{++}) or magnesium (Mg^{++})] centrifuging each time at 350 *g* for 10 min at 4°C. EDTA anticoagulates by chelating calcium ions. The PBS was, therefore, Ca^{++} and Mg^{++} free in order to prevent clot formation in the mononuclear cell layer. As the PMN layer usually contained red blood cells (RBC) it was treated with RBC lytic buffer as follows:- the PMN were resuspended in 7 ml of the lytic buffer (NH_4CL 8.28 g, $NaHCO_3$ 0.4 g, EDTA-disodium salt 0.0336 g, made up to one litre in distilled water) and the tube kept on ice for 6 min. The cells were then washed twice in PBS (Ca^{++} and Mg^{++} free) and the cell pellets resuspended in 2 ml of Hanks' balanced salt solution (HBSS) ready for counting. The number of cells obtained using this technique, isolated from the patients in the 3 clinical groups is shown in Table 2.2. The purity of the cell populations is shown in Table 2.3.

2.6 Cell Counts and Cell Viability

2.6.1 White blood cell counts

Cell suspensions were diluted 1:20 in Turk's fluid (2% acetic acid plus trace gentian violet), mixed and left for 5 min before being counted in an improved Neubauer counting chamber[236]. The peripheral white blood cell counts from the blood of the people in the 3 clinical groups is shown in Table 2.4.

2.6.2 Cell viability

A cell viability was assessed using the trypan blue exclusion method[237]. The cells were diluted 1:2 with 0.4% trypan blue (Gibco) and 100 cells were counted. The viable cells, those that excluded trypan blue were expressed as a percentage of the total.

2.7 Preparation of Cytocentrifuge Smears

A monolayer of cells was prepared by adding 50 µl of 5 x 10^6/ml cell suspension to 0.3 ml saline in a cytocentrifuge cup and spinning at 375 *g* for 5 min in a cytocentrifuge (Shandon Southern Products Ltd, Cheshire). The slide was then removed and allowed to dry before being fixed. Preparations of the cells are illustrated in Figs 2.3, 2.4.

2.8 Cell Staining and Differential Counts

All slide preparations (cytocentrifuge smears, blood films) were fixed for 5 min in methanol (BDH Chemicals Ltd, Poole, England) before being stained using the following May-Grünwald Giemsa staining method. The slides were stained in May-Grünwald stain (0.6 g into 100 ml of methanol warmed to 60°C and then left overnight and filtered for 10 min) and then in Giemsa stain (diluted 1:15 in 0.066 M Sorensen's buffer) for 10 min rinsed 2 x 5 min in pH 6.8 buffer and allowed to dry. The number of different leukocyte types in one hundred cells were counted and the result expressed as a percentage and in absolute numbers[238]. The cell differentials of the white blood cells from patients in the clinical groups are given in Table 2.5.

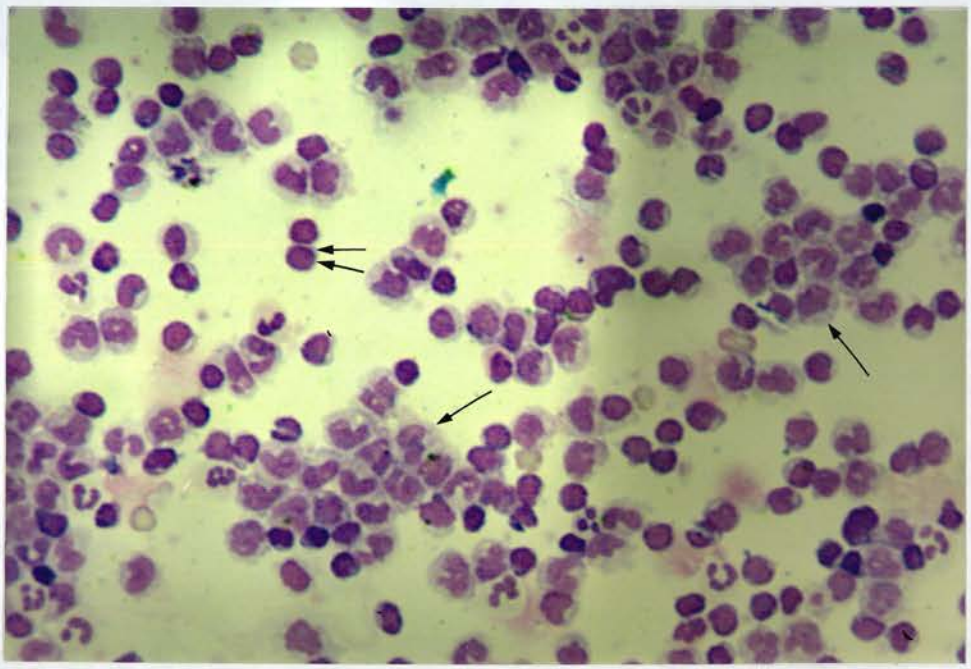


Figure 2.3 Cytocentrifuge smear of human mononuclear cells isolated by density gradient centrifugation. The cells illustrated are monocytes (→) and lymphocytes (⇉). Original magnification x 400.

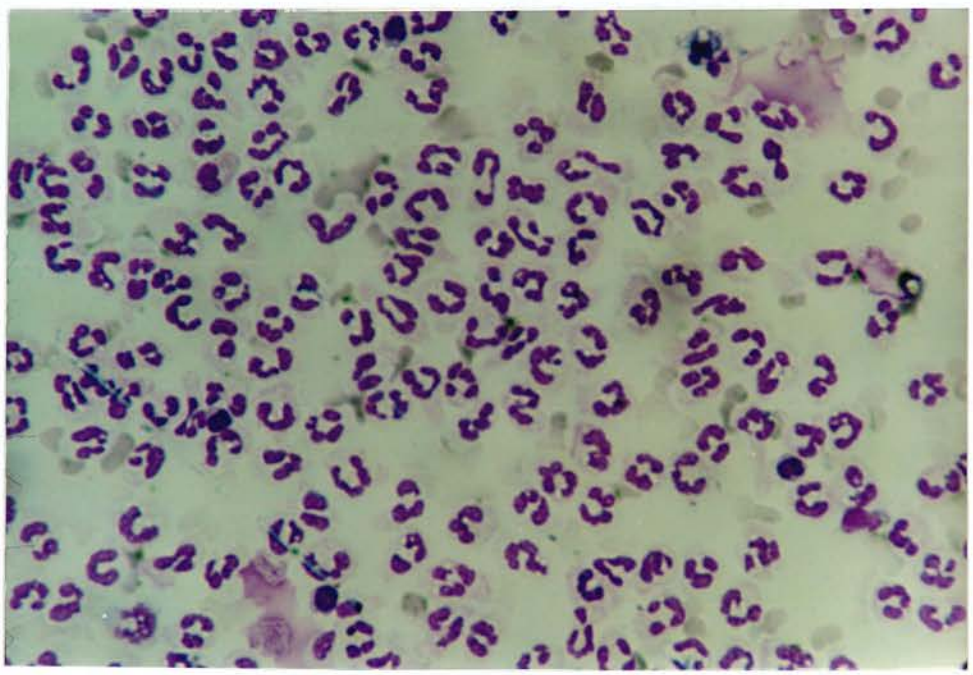


Figure 2.4 Cytocentrifuge smear of human granulocytes isolated by density gradient centrifugation. The preparation contains >92% PMN. Original magnification x 400.

TABLE 2.5 Differential white blood cell counts of subjects divided by clinical group

Clinical Status	Number in Group	DIFFERENTIAL % PMN	Lymphocyte	Monocyte	Eosinophil	Basophil
Control	15	59.07 (12.7)[1]	33.2 (11.0)	4.87 (1.9)	2.73 (1.9)	0 (0)
Asthma Non-sensitised	14	56.21 (11.3)	33.93 (11.9)	4.71 (3.6)	5.07 (4.3)	0.07 (0.27)
Asthma Sensitised	13	56.46 (11.7)	33.08 (11.1)	6.15 (2.0)	4.38 (4.2)	0 (0)

1 Mean (SD)

2.9 Serum Separation and Treatment

Clotted blood was spun at 400 *g* for 20 min in an MSE super minor centrifuge at room temperature. The serum was then removed and divided into three samples which were treated in the following way:

(i) stored at 4°C,

(ii) heated at 50°C in a water bath for 20 min

(iii) heated at 56°C in a water bath for 30 min

The sera were used in the assay systems within one hour of treatment. Heat inactivation is conventionally used to examine the effects of complement in phagocytosis/killing assay systems. Storage at 4°C for short periods preserves heat labile complement components. Heating for 20 min at 50°C destroys factor B which is involved in the alternative pathway of complement activation. Heating at 56°C for 30 min destroys all other heat labile complement components including those which are involved in the classical as well as the alternative pathway of complement activation[239].

2.10 Detection of Serum Antibody to A. fumigatus

The presence of serum antibody to A. fumigatus was estimated using counter immunoelectrophoresis (CIEP) which detects mainly IgG precipitating antibodies, and the radio-allergosorbent test (RAST) for the determination of circulating specific IgE antibodies.

2.10.1 CIEP

The method used was based on an established procedure[240]. Agarose (1% Sigma) was dissolved in 0.05 M sodium barbitone buffer pH 8.6

(10.31 g sodium barbitone, 12 ml $\bar{N}$ HCL into one litre of distilled water). A volume of 8 ml of 1% agar in sodium barbitone buffer was added to glass plates (3 x 4 in) and the plates allowed to set at $4^{o}C$ for 6 h. The wells were punched and the agar allowed to settle overnight. The test sera were added to the top wells while the A. fumigatus antigens and control added to the lower wells. The plates were electrophoresed at a constant voltage of 150 Volts for 1.5 h using a Shandon (Shandon Southern Products Ltd, Runcorn, Cheshire) power pack. After this time the plates were removed and put into a moist box and left at room temperature for 18 h. The plates were put into saline for one hour to remove non-bound protein, then rinsed in distilled water, and allowed to dry with a piece of filter paper on top. The plates were stained using Amido Schwartz stain (50 g mercuric fluoride was dissolved in 950 ml distilled water by gentle heating, 5 g Napthalene black was added to 50 ml of glacial acetic acid and shaken for 30 min and then added to the mercuric chloride solution and filtered through filter paper to remove any deposit) for 30 min and then cleared using methanol acetic acid (9:1) until background staining was removed. Positive staining came up as a sharp blue line between the sera and the antigen preparation. Positive and negative controls were included in each run. A typical example is illustrated in Fig 2.5.

2.10.2 RAST

This test was kindly carried out by Dr Hugh McFarlane of the Department of Dermatology, University of Edinburgh using the Phadebas RAST test kit supplied by Pharmacia Diagnostics, Sweden. The

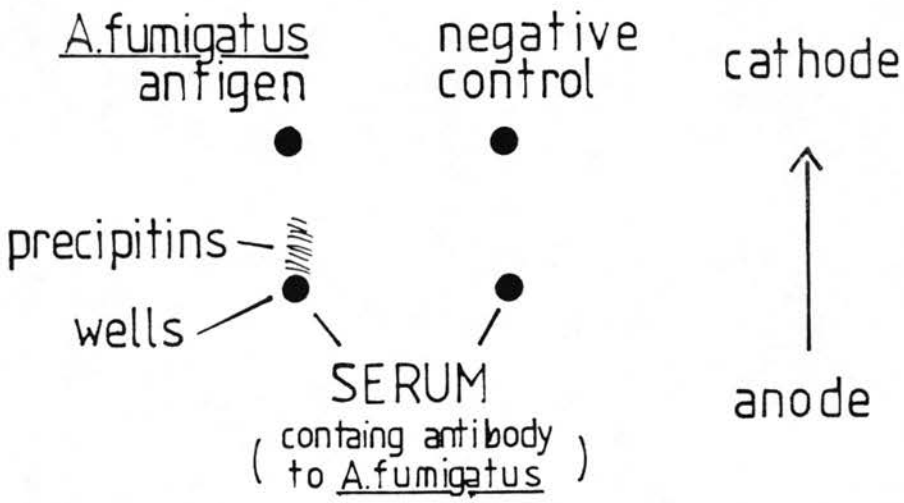


Figure 2.5 An example of the precipitation lines that occur when serum containing specific antibody to A. fumigatus is electrophoresed against A. fumigatus antigens when compared with control solution (negative control). Direction of current (↓).

principle of the test involves incubating the patient's serum with special discs coated with A. fumigatus antigen. Any specific IgE antibodies in the patient's serum bind to the disc to form a disc-allergen-IgE complex. Sepharose beads coated with radio-labelled anti-human IgE sera are then incubated with the discs and any specific A. fumigatus IgE antibody present in the patient's sera will bind to the radio-labelled sepharose beads to form a disc-allergen-IgE-radio-labelled-anti-IgE complex which is measured in a gamma counter[241].

2.11 Animals Used in the Study

The rodents used in the study were an important source of cells and serum. The animals used were syngeneic C57Bl/6 mice and syngeneic PVG hooded rats. The animals were obtained from the IOM Animal Breeding Unit, Bush House Estate.

2.12 Animal Maintenance

The animals were housed in the Animal Unit at the City Hospital, Edinburgh. They were maintained in plastic cages on grade 6 sawdust bedding. The animals were fed standard laboratory rat and mouse diet and tap water ad libitum. The animal house was mechanically ventilated and illuminated on a 12 h photoperiod at an average temperature of 22°C.

2.13 Intraperitoneal Injection of Mice

Peritoneal exudate cells were obtained from the peritoneal cavity of untreated naive mice or following intraperitoneal injection of

thioglycollate, a non-specific macrophage eliciting agent[242] or Corynebacterium parvum which has been shown to activate macrophages as well as having immunomodulatory effects[243]. In addition mice were challenged with suspensions of fungal spores. All animals were used at between 12 and 20 weeks of age at the time of injection.

Thioglycollate was prepared by boiling 3 g of thioglycollate medium (Gibco Europe Ltd) in 100 ml of distilled water. The solution was then autoclaved at 15 lb/in^2 at 121^{o}C for 3 min. After cooling the thioglycollate was put into aliquots in sterile containers and stored in the dark for 3 months prior to use. Storage renders the thioglycollate more effective in increasing the yield of peritoneal macrophages[242]. A volume of 0.5 ml was used for injection and the animals left for 4 days prior to harvesting.

A volume of 200 µl (1.4 mg) of heat killed C. parvum (Wellcome Biotechnology Ltd) was used for injection and the animals left for 5 days prior to harvesting. All animals were injected in the mid-peritoneal cavity beneath the line of the liver and above the bladder.

2.14 Harvesting of Peritoneal Exudate Cells

When required the mice were killed by ether overdose and the peritoneal exudate cells harvested. An incision was made into the midline of the peritoneal cavity of the mouse, the fur pulled back and 5 ml of HBSS usually containing 10 units/ml heparin (preservative-free) were injected into the peritoneal cavity. The peritoneum was then gently massaged and the mouse left for one minute. An incision

was made into the middle of the skin covering the peritoneal cavity and the animal flipped over a cold glass filter funnel which was placed in a plastic universal container in an ice bucket. This was a convenient method which allowed the peritoneal fluid to drain from the peritoneal cavity without requiring the use of a syringe or multiple washes. The washouts containing the cells were kept on ice and spun at 4°C at 400 *g* for 8 min. All cells were washed once in HBSS prior to counting. The differential cell counts obtained are shown in Table 2.6.

2.15 Mouse Model of Immunisation

To obtain an animal model of systemic sensitisation to A. fumigatus an antigenic extract of A. fumigatus was used. The extract was obtained from Dr J Edwards, MRC, Sully Hospital, Penarth who provided a sensitisation schedule. The A. fumigatus extract was prepared by Dr Edwards as follows: (Method description written by Dr J Edwards) "An A. fumigatus strain No. I355 was grown by the double dialysis method[244] with added glucose at 10 g/l for 7-10 days at 30°C. The mat was removed and washed (saline/azide) and extracted by Hughes Press or equivalent. Hughes Press entailed freezing and squeezing through a small orifice to give a decompression rupture. After centrifuging, the debris was removed and the supernatant dialysed against running tap water. Concentration to 25-40 mg/ml (dry weight) was by air dialysis and storage was at -20°C. The extracellular material was dialysed against running tap water, concentrated by air dialysis and centrifuged. The soluble material was concentrated to 25-40 mg/ml and kept at -20°C. The extract used contained 50:50 somatic and

TABLE 2.6 Differential cell counts of mouse peritoneal exudate cells following injection with thioglycollate or *C. parvum*

Treatment	Differential %			
	Macrophage	PMN	Lymphocyte	Eosinophil Mast Cell
None	93.2 (2.0)[1]	0.8 (1.0)	3.8 (1.5)	2.2 (1.5)
Thioglycollate	92 (1.4)	2.3 (1.4)	3.3 (2.5)	2.3 (2.1)
C. parvum	63 (10.9)	34.2 (11.4)	2.6 (1.8)	0.5 (0.8)

1 Mean (SD) of counts from a pool of 6 mice from 6 separate experments

culture filtrate having been thawed, spun and filtered through a <450 mμ filter. Dry weight was determined before dispensing and the volume adjusted to 20 mg/ml."

The animals were injected intramuscularly into the hind leg with 0.05 ml of 0.5 mg of A. fumigatus antigen in 0.25 mg Freund's complete adjuvant. After 2 weeks the animals were boosted subcutaneously at the scruff of the neck with 0.5 mg A. fumigatus extract. After a further one, 2 and 3 weeks blood was obtained by cardiac puncture and the sera tested for specific antibody using CIEP.

2.16 Intratracheal Instillation of Rats

This procedure was performed by Dr K Donaldson of the I.O.M. PVG rats were anaesthetised with ether and the trachea exposed by blunt dissection. A small incision was made into the trachea and a blunt ended needle was introduced down to the carina. A volume of 0.1 ml containing 1.4 mg of heat killed C. parvum was injected and the skin closed with metal clips. To obtain an acute PMN-rich exudate the rats were sacrificed 16 h later. The cell population comprised >90% PMN.

2.17 Lung Lavage Technique

PVG rats were sacrificed by intraperitoneal injection of pentobarbitone sodium. The rats were dissected to expose the lungs and the trachea. The trachea was raised slightly and a small hole made between the cartilage rings. A cannula made from a blunted 16 g needle was inserted and the needle tied in position with fishing line.

The lungs, heart, trachea and needle were carefully removed from the thoracic cavity and the lungs slowly inflated with 10 ml of warm saline (37°C). The saline was then slowly withdrawn from the lungs and decanted into plastic tubes which were kept on ice. This procedure was repeated four times. The cells were centrifuged at 350 *g* for 10 min at 4°C and the pellet resuspended in ice cold HBSS prior to counting as previously described.

2.18 Isolation of Human Pulmonary Macrophages

Human pulmonary macrophages were obtained from fresh surgically resected lung specimens. Sections of peripheral lung tissue were taken by Dr K Kerr of the Department of Pathology, from macroscopically 'normal' areas when available, in non-diseased lobes and always avoiding areas of collapse, infection or endogenous lipoid pneumonia. The tissue was scissored into small fragments and filtered through a 60 µm wire mesh with ice cold HBSS. The cell suspension was spun at 375 *g* for 10 min and the cells resuspended in medium (RPMI-Flow Laboratories, Irvine, Scotland). A cytocentrifuge smear was prepared and a differential cell count performed. A typical smear is illustrated in Fig 2.6.

2.19 Purification of Macrophages by Adherence

One hundred microlitres of a cell suspension containing 5 x 10^6/ml macrophages were added to flat-bottomed Removawells (Dynatech) or glass coverslips 6 x 22 mm (Chance Propper Ltd, Warley, England). The cells were allowed to adhere at 37°C in a 5% CO_2 incubator for one hour. After this time the cells were washed twice in warm (37°C)

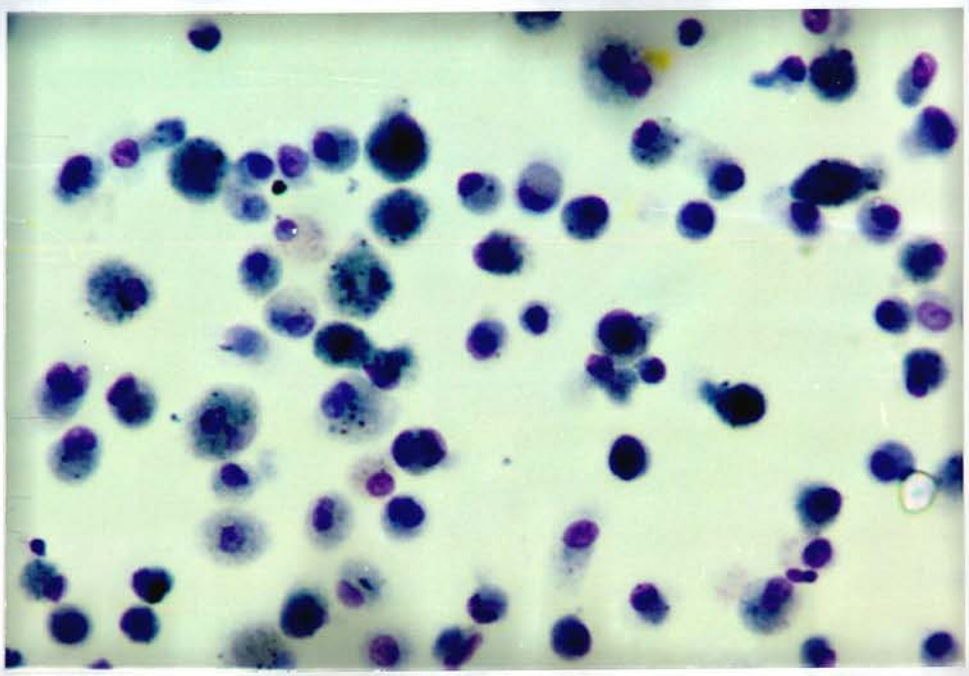


Figure 2.6 A photomicrograph of a cytocentrifuge smear of human pulmonary macrophages, obtained from human lung tissue. Original magnification x 400.

RPMI prior to use.

2.20 Fungal Spores

All fungal spores used in the study were prepared and maintained by Dr L.J.R. Milne, Mycology Unit, Western General Hospital, Edinburgh.

2.20.1 Aspergillus fumigatus

The principal strain of A. fumigatus used was an isolate grown from the sputum of a patient with allergic bronchopulmonary aspergillosis (Fig 2.7). In addition, for some of the work, multiple isolates of A. fumigatus obtained from the sputum of a patient with aspergilloma were used. A batch of A. fumigatus was prepared by culturing the spores for two weeks at 37°C on 4% malt-peptone-agar (40 g malt extract (The Boots Company, Nottingham, England), 5 g bacteriological peptone (Oxoid, Basingstoke, England), 15 g agar (Gibco) dissolved in one litre of distilled water (pH 5.6) and autoclaved at 121°C for 15 min). The spores were obtained from the cultures by tapping the plates gently to release the spores which were then distributed into sterile glass bijoux and kept at room temperature until required. Once a week spores were removed from the stock supply and cultured at 28°C on malt-peptone-agar slopes to obtain spores for use in the experiments.

2.20.2 Penicillium ochrochloron

The strain of P. ochrochloron was IMI 61531 (Fig 2.8). The freeze-dried sample was put into distilled water, divided into aliquots in sterile dry glass bijoux and stored at 4°C. Once a week, an aliquot of the suspension culture of P. ochrochloron was removed from the

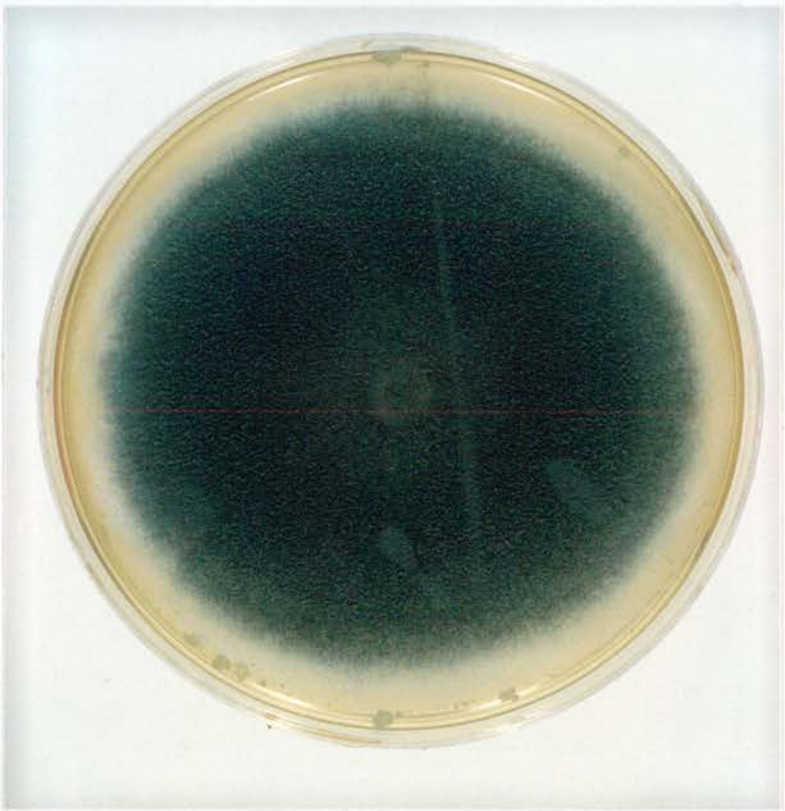


Figure 2.7 A colony of A. fumigatus (the strain used in this study) grown at 28°C for 5 days on malt-peptone agar.

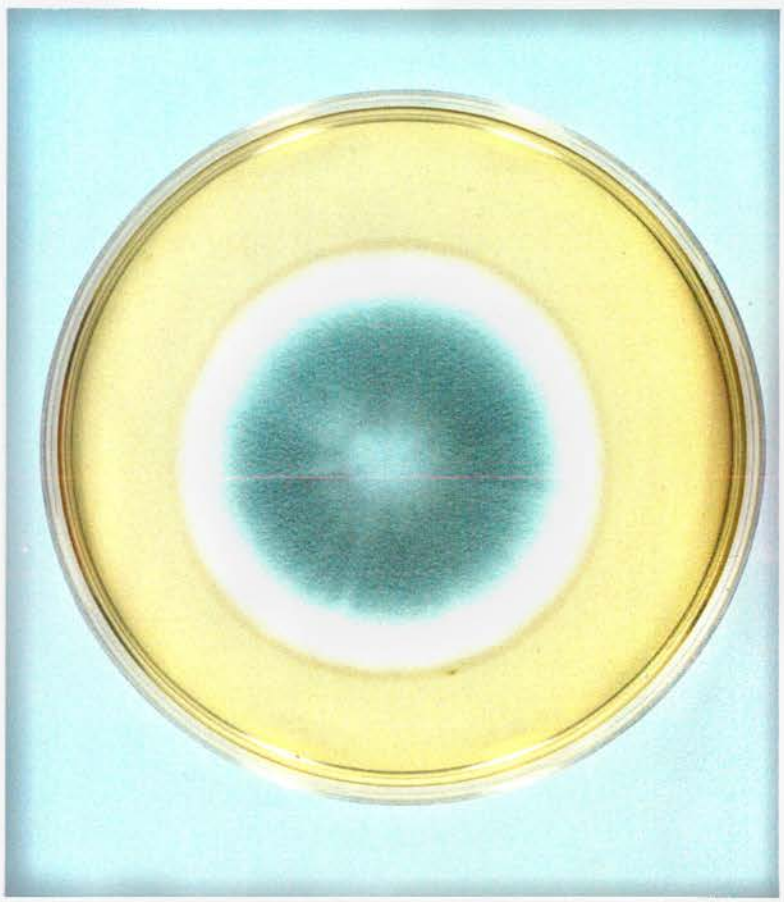


Figure 2.8 A colony of P. ochrochloron (the strain used in this study) grown at 28°C for 5 days on malt-peptone agar.

stock supply and cultured at 28°C on malt-peptone-agar slopes to obtain spores for use in the experiments.

2.21 Harvesting of Fungal Spores

Fungal spores were removed from the agar slopes using a sterile swab (Medical Wire and Equipment Co. Ltd, Corsham, England) and transferred into 5 ml of HBSS-0.01% bovine serum albumin (BSA). The spores were dispersed manually using a glass homogeniser (Gallenkamp, East Kilbride, Scotland). The dispersed spores were filtered through nylon mesh which had a pore size of 5 µm. Filtration removed any mycelial fragments, chains of spores or spore clumps to yield a spore suspension containing only single spores. The spores were diluted 1:200 in HBSS-0.01% BSA and counted in an improved Neubauer chamber. All spores were freshly prepared before each experiment and kept on ice until required.

2.22 Spore Diffusates

Single spore suspensions were prepared as previously described (2.21) In addition spores were prepared in distilled water or HBSS alone. All spores were adjusted to a concentration of 10^8/ml and incubated at 37°C for up to 3 h. After incubation the tube containing the spores was spun at 500 *g* for 5 min and the supernatant containing the diffusate filtered through a 0.22 µm sterile filter (Flow Laboratories). The diffusate was stored at -70°C until required.

2.23 Preparation of Zymosan

Zymosan (Sigma) a cell wall extract of the yeast, Saccharomyces cerevisiae was used as an additional control for some of the assays. Zymosan was prepared by dissolving 5 g in PBS which was then boiled for 5 min. The boiled zymosan was washed x 2 in HBSS by centrifugation at 400 *g* for 10 min and adjusted to 20 mg/ml in HBSS. Aliquots were stored at -70°C until required.

2.24 Opsonisation of Spores and Zymosan

The spores and zymosan were opsonised in 5% sera for 30 min at 37°C immediately prior to use. For some of the assay systems (production of reactive oxygen intermediates) the spores were washed in HBSS, by centrifugation at 400 *g* for 10 min and then resuspended in HBSS alone. This step was required in order to remove the excess serum which is thought to interfere with these particular assays. For the phagocytosis/killing assay, spores were opsonised in RPMI-5% sera and then used directly in the assay (without washing). All opsonised spores were kept on ice until required.

2.25 Estimation of Cell Association of Fungal Spores

A volume of 50 µl of human PMN or mononuclear cells (1×10^7/ml in RPMI-5% serum) was added to flat bottomed microtitre plates (Gibco). Wells containing 5×10^5 adherent macrophages were prepared as previously described (2.19). At the same time control wells containing 50 µl of RPMI-5% serum alone were set up. One hundred microlitres of opsonised spores (10^7/ml in RPMI-5% serum) were added

to give a final spore:phagocytic cell ratio of 2:1 or 1:1. The results of a standardisation experiment to determine optimum spore:cell ratio is illustrated in Fig 2.9. To increase contact between spores and cells the microtitre plates were centrifuged at 125 *g* for 5 min and then incubated at 37°C for one hour. After this time the sedimented non-cell associated spores were resuspended by gently mixing and an aliquot of supernatant (50 µl) was removed and diluted 1:2 in saline/Trypan blue (W/V 1:1) and the number of free spores present counted using an improved Neubauer counting chamber. Total spores were estimated from the number of spores in the supernatant of the control wells containing spores alone, and this was used as a baseline to determine the percentage of spores becoming cell-associated using the following formula:

$$\%\ \text{of spores cell-associated} = \frac{(\text{total spores} - \text{spores non-cell-associated})}{\text{total spores}} \times 100$$

All experiments were carried out in triplicate. The basic procedure used in this technique is illustrated in Fig 2.10.

2.26 Phagocytosis of Radiolabelled Antibody Coated Sheep Red Blood Cells

This method of phagocytosis was used to discriminate between attached and ingested sheep red blood cells (srbc). All non-ingested srbc are removed by lysis, thereby permitting an accurate estimation of ingestion.

The method used was based on that described by Shaw and Griffin[245]. The srbc in Alsever's solution (Tissue Culture Services Ltd, Slough,

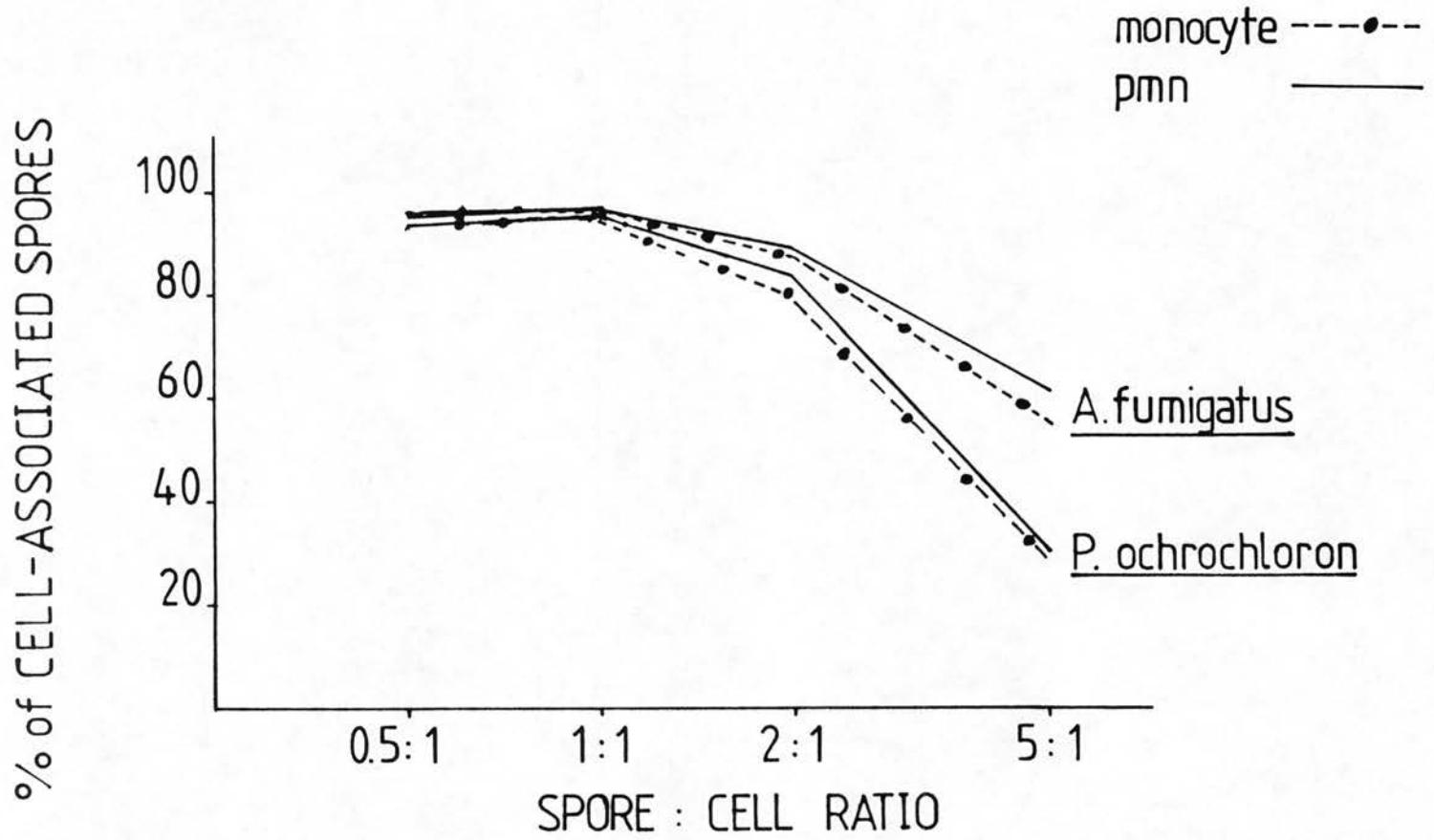


Figure 2.9 The effect of increasing spore:cell ratios (0.5:1- 5:1) on the cell-association of spores of A. fumigatus and P. ochrochloron, opsonised in 5% pooled normal sera, with human monocytes and PMN following incubation for 1 h at 37°C. The results are expresssed as the mean percentage of spores becoming cell-associated, obtained from two experiments.

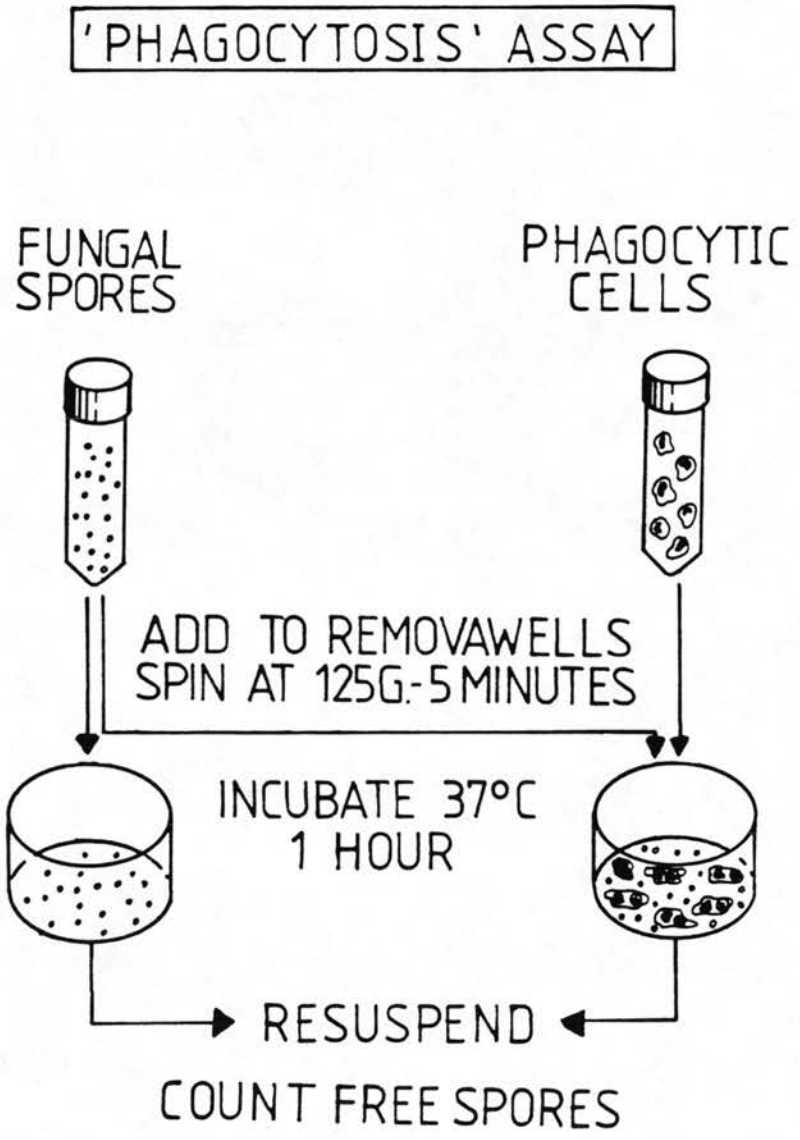


Figure 2.10 Illustration of the basic procedure used for the estimation of phagocytic cell-association of fungal spores.

England) were washed x 3 in PBS. The washed packed srbc were radio-labelled by incubation in ^{51} sodium chromate (^{51}Cr) 10^8 (Amersham International PLC, Aylesbury, England). A volume of 10^8 srbc/100 µ Ci ^{51}Cr were incubated for one hour at 37°C. The ^{51}Cr srbc were washed once in PBS and adjusted to 10^9 ^{51}Cr srbc/ml. This suspension was then divided into two aliquots and the following added:

(i) Irrelevant antibody (mouse IgG; Sigma) at 10 µg/10^7 srbc

(ii) Monoclonal antibody to srbc (mouse IgG anti-srbc, Sera-Lab Ltd, Crawley Down, England) at 10 µg/10^7 srbc.

These mixtures were incubated for 15 min at 37°C then washed twice in ice-cold PBS by centrifuging at 300 *g* for 10 min. The antibody coated ^{51}Cr srbc were then suspended in RPMI-20% heat inactivated foetal calf serum (Gibco) at a concentration of 4 x 10^7/ml. C. parvum stimulated mouse peritoneal exudate cells was added to flat-bottomed Removawells at a concentration of 2 x 10^5/ml in 100 µl of RPMI-10% heat inactivated foetal calf serum. The cells were left to adhere for 3 h at 37°C in 5% CO_2 and then washed twice in warm HBSS (37°C). Five separate treatments were used and so 50 µl of one of the following solutions were added to Removawell cultures.

(i) HBSS alone

(ii) HBSS alone

(iii) A. fumigatus diffusate,

(iv) P. ochrochloron diffusate,

(v) a known inhibitor of phagocytosis - cytochalasin B at 25 µg/ml (Sigma).

To treatment (i) 50 µl of ^{51}Cr srbc coated in irrelevant antibody

was added whilst treatments (ii) - (v) 50 µl of ^{51}Cr srbc coated with mouse IgG anti-srbc was added.

The Removawells were centrifuged for 5 min at 125 *g* to enhance contact then incubated at 37°C in 5% CO_2 for 1.5 h. The supernatant was removed and 100 µl of ice-cold red cell lytic buffer added to lyse non-phagocytosed srbc. After 5 min the lytic buffer was removed and the cell washed x 3 in cold PBS. The Removawells containing cells with phagocytosed ^{51}Cr srbc were counted in a gamma counter (LKB-Rack Gamma II). For each experiment 5 replicates were performed.

2.27 Spore Killing Assay

This assay is based on the estimation of colony forming units following incubation of fungal spores with phagocytic cells for 3 h at 37°C illustrated in Fig 2.11. A volume of 50 µl of human PMN or mononuclear cells (1 x 10^7/ml) in RPMI-5% autologous serum) were added to flat bottomed Removawells. Removawells containing 5 x 10^5 adherent macrophages were prepared as previously described (see sections 2.19 & 2.25). At the same time control wells containing 50 µl of RPMI-5% serum alone were set up. A volume of 100 µl of appropriately opsonised spores (5 x 10^6/ml in RPMI-5% serum, see section 2.24) were added to all wells to give a final spore:phagocytic cell ratio of 2:1 and 1:1. The results of standardisation experiments to determine optimum spore:cell ratios are illustrated in Fig 2.12. To increase contact between spores and cells the Removawells were centrifuged at 125 *g* for 5 min. The cells plus spores were then incubated in a humidified chamber for 3 h at 37°C. After this time the wells were harvested by transferring the Removawells to sterile conical tubes

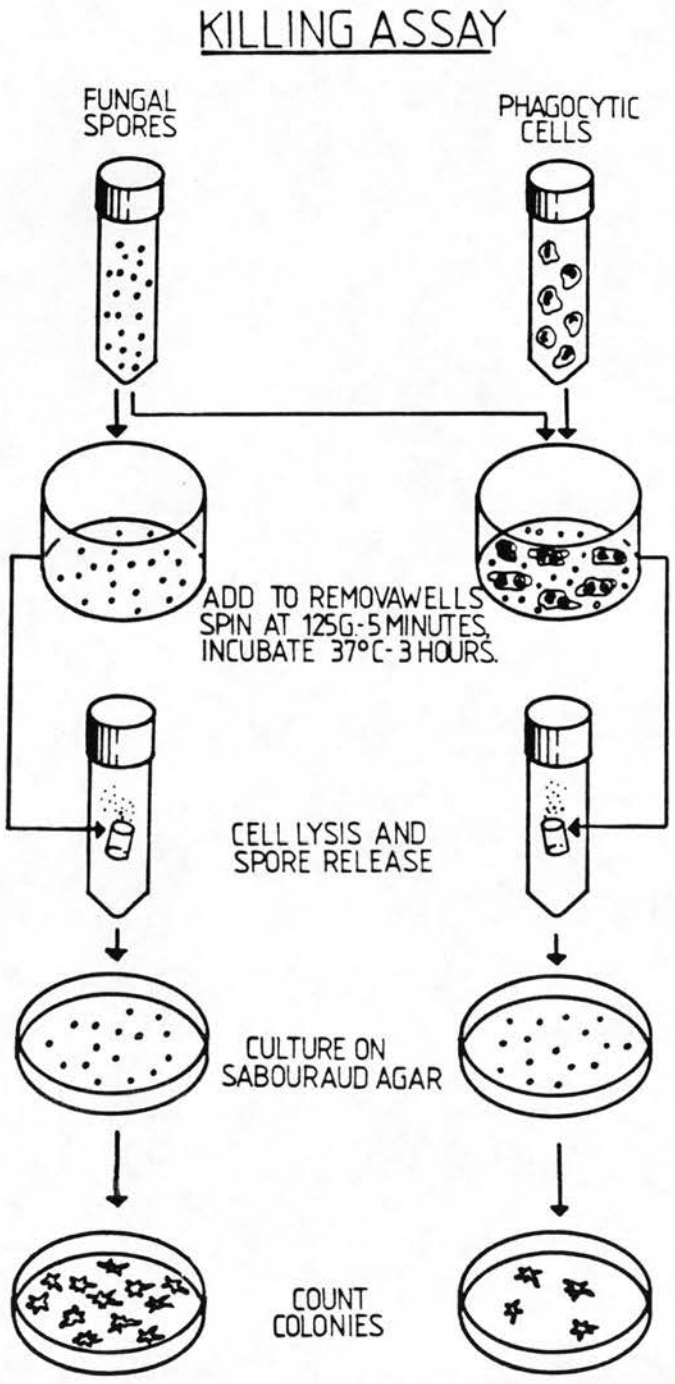


Figure 2.11 Illustration of the basic procedure used for the estimation of phagocytic cell killing of fungal spores.

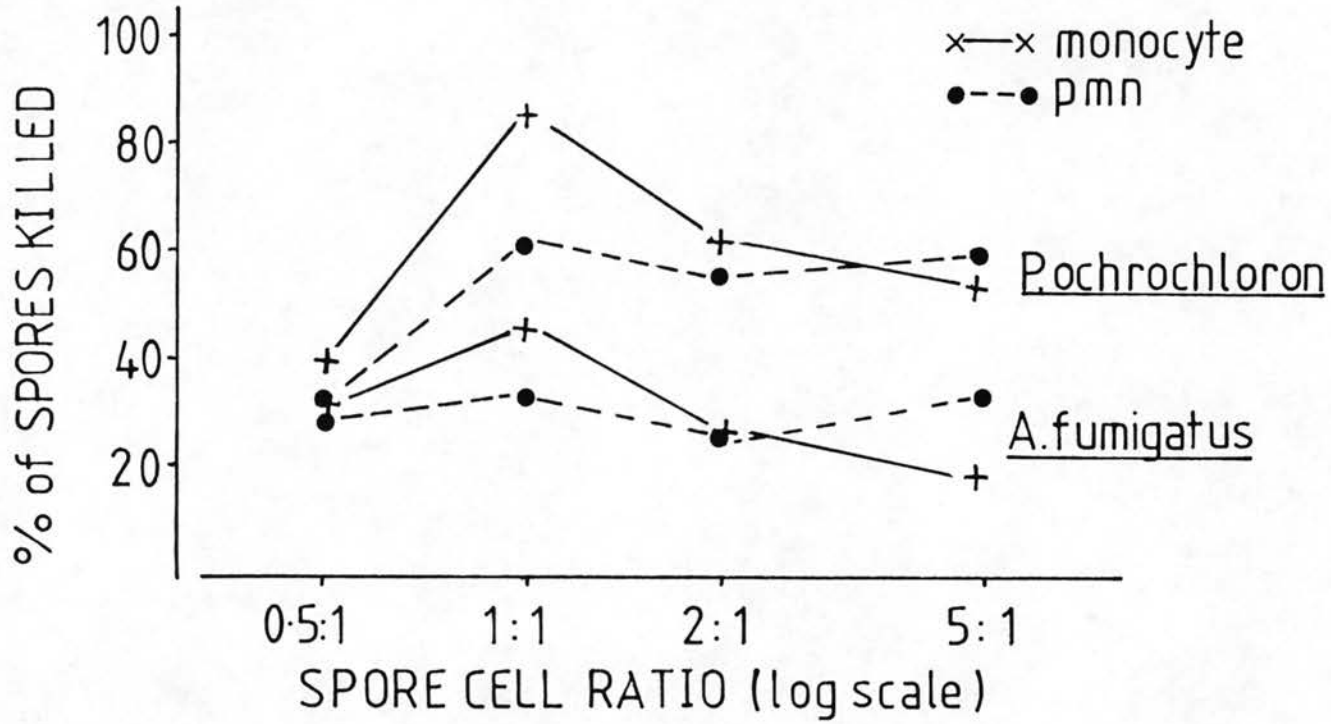


Figure 2.12 The effect of increasing spore:cell ratio (0.5:1 - 5:1) on the ability of human monocytes and PMN to kill spores of A. fumigatus and P. ochrochloron which had been opsonised in 5% pooled normal sera, following incubation at 37°C for 3 h. Results expressed as the mean percentage of spores killed, obtained from two experiments.

(Sterilin) containing distilled water-0.1% Triton-X (Sigma) which were mixed by vortexing on a Whirlimix. This process causes disruption of the cells allowing release of the cell-associated fungal spores. After 5 min a 50 µl aliquot was transferred to a sterile bijou containing 3.95 ml of PBS-0.01% BSA from which a 125 µl sample was plated out using a sterile inoculating loop (Nunc) on to a Petri dish of Sabouraud dextrose agar (65 g of Sabouraud dextrose agar (Oxoid) dissolved in one litre of distilled water and autoclaved at 121°C for 15 min - Sabouraud plates were kindly prepared by the Bacteriology Department at the City Hospital). The plates were incubated at 37°C for 24-40 h (A. fumigatus-24 h, P. ochrochloron-40 h; optimum times for colony growth). The colony forming units (CFU) were counted using a semi-automated colony counter (Chiltern) and the results are expressed as:

$$\text{The percentage of spores killed} = \frac{(\text{Control CFU-cells/spores CFU})}{\text{Control CFU}} \times 100$$

All experiments were set up in triplicate.

A considerable amount of time was spent on the development and standardisation of this spore killing assay. Numerous technical problems had to be overcome, the primary one being adaptations to take into account the biological behaviour of the fungal spores. Initial studies revealed that the ability of the phagocytic cells to kill fungal spores was dependent upon the spore's state of germination, prior to challenge. If spores were harvested (0 h spores) opsonised and then incubated with mouse peritoneal exudate cells for one hour instead of the spores being killed there was an increase in CFU (% surviving) relative to the control spore suspension. However, if the

spores were harvested then preincubated for 3 h at 37°C (3 h spores) prior to opsonisation the percentage of spores surviving in relation to the control spore suspension was substantially reduced (Table 2.7). This result suggested that spores in early stages of germination (not visible) are more susceptible to killing when compared with dormant spores. In order to try to explain the reasons for this enhanced germination the following experiments were carried out.

2.27.1 Effect of incubation time on CFU formation

The spores of A. fumigatus were obtained by shaking the free spores from the culture as it was thought that these 'released' spores were similar to those that would normally be inhaled by man. The possibility that these released spores would be in a fairly dormant phase (thereby accounting for the low CFU in the control suspension), and that incubation at 37°C in suspension, may bring them out of this phase, was considered. Suspensions of spores of A. fumigatus and P. ochrochloron were incubated at 37°C, samples were taken at set time points, plated out, incubated overnight and CFU estimated. It can be seen from Fig 2.13 that the CFU of both A. fumigatus and P. ochrochloron increased by approximately 25%, although this increase plateaued much earlier for P. ochrochloron (2 h) than for A. fumigatus (5 h).

Although these results partially explained the increase in CFU found between using 0 and 3 h spores another experiment was carried out to test if cell products may also be contributing to the enhanced germination.

TABLE 2.7 Effect of preincubation at 37°C on the percentage of opsonised spores of A. fumigatus surviving after incubation with mouse peritoneal exudate cells for one hour at a spore : cell ratio of 1:1. The % is calculated from the CFU of the control spores alone.

Preincubation (h)	% Spores added surviving
0	152 (18.8)[1]
3	101 (16.1)

1 Mean (SD) of eight experiments

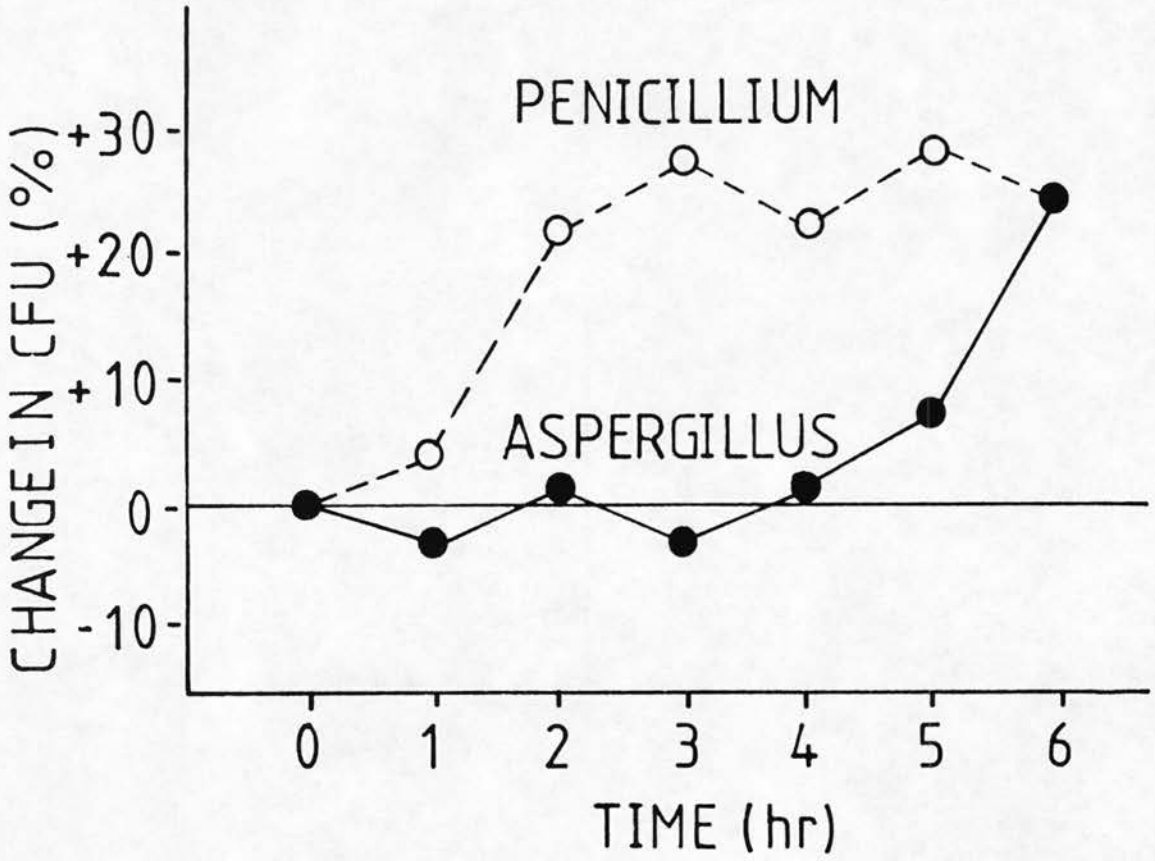


Figure 2.13 The effect of incubating spores of A. fumigatus and P. ochrochloron for up to 6 h in phosphate buffered saline containing 1% bovine serum albumin on the growth of colony forming units (CFU). A fixed volume of spores was sampled every hour and plated on Sabouraud agar. The results for each of the time points are expressed as the percentage difference (change) obtained when compared with the CFU for non-preincubated (0 h) spores.

2.27.2 Effect of cell products on numbers of CFU

Adherent mouse peritoneal exudate cells in medium containing 5% serum were subjected to 3 treatments:

(i) Supernatants (containing resting cell products) were obtained from cells which had been incubated at 37° for one hour.

(ii) Supernatants were obtained from cells which had been frozen, thawed and then spun.

(iii) Cells were treated with latex beads for one hour in order to stimulate phagocytosis. The supernatants were then removed.

The supernatants removed from these 3 treatments were incubated with 0 h spores for one hour at 37°C and CFU estimated. When compared with control alone in medium containing serum it was found that these 3 treatments enhanced the germination of the 0 h spores by approximately 40% (see Table 2.8). Although it was only one experiment this result indicated that cell products obtained from resting macrophages were sufficient to enhance the germination of spores.

Further work was undertaken to see if phagocytic cells needed a longer incubation period in which to kill the spores. The results shown in Fig 2.14 demonstrate that incubating 0 h spores with mouse peritoneal exudate cells for up to 4 h resulted in a time-dependent increase in spore killing.

The overall results of these studies showed that in order to obtain reproducible results it was very important to always have the spores

TABLE 2.8 The effect on the germination of spores of *A. fumigatus* following the addition of supernatants of mouse peritoneal exudate cells, which had been subjected to three treatments

Treatment (Supernatant)	Percentage difference from control CFU (Mean [SD])
None (resting cells)	+ 38 (2.5)
Frozen and thawed	+ 45 (2.3)
Latex treated	+ 43 (5.3)

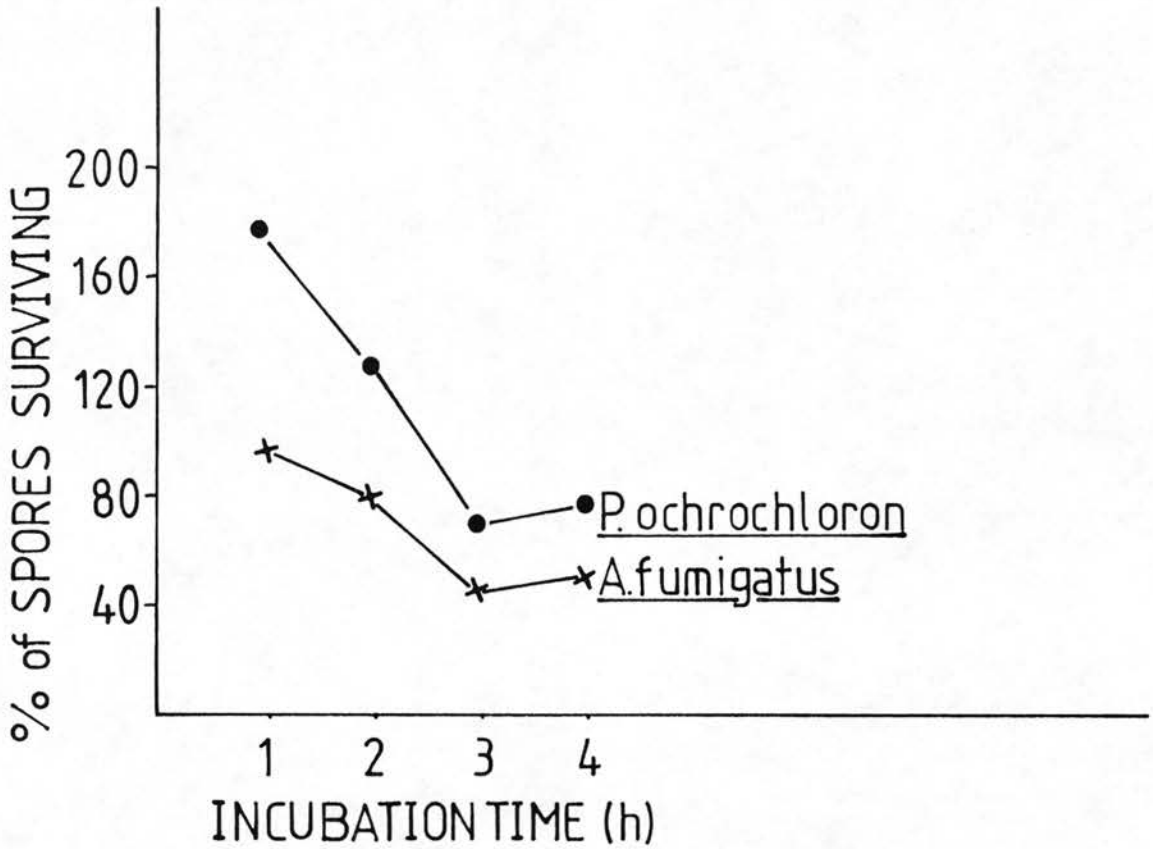


Figure 2.14 The effect of the incubation time (1 h - 4 h) on the ability of mouse peritoneal exudate cells to kill spores of A. fumigatus and P. ochrochloron, opsonised in 5% autologous serum. Results expressed as the mean percentage of spores killed.

in the same phase of growth at the beginning of each assay (see 2.20). It was also decided that a 3 h incubation period would be used for all spore-killing experiments.

2.28 Preparation of Cell/Spores for Microscopy

Phagocytic cells (200 µl of 5 x 10^6/ml in RPMI-5% autologous serum) were allowed to adhere to glass microchamber slides (LAB-TEK, Miles Laboratories) or glass coverslips (13 mm diameter) for one hour at 37°C in 5% CO_2. The cultures were then gently rinsed twice in warm HBSS (to remove non-adherent cells). Opsonised spores in RPMI-5% autologous serum were added to the adherent phagocytic cells at a spore:cell ratio of between 2:1 and 1:1 and the cultures incubated at 37°C and 5% CO_2 for 1.5 h. Spores alone were added to coverslips which had been pre-treated with 1% alcian blue (Sigma) to increase the surface charge of the glass, which results in increased attachment of particles. After this period, excess spores were removed by gently washing 3 times in warm HBSS the slides were fixed in methanol and stained in May Grunwald Giemsa as previously described. For examination by either Phase contrast or Nomarski differential interference optics (Carol Zeiss, West Germany), the slides were mounted in HBSS. The coverslips were processed for scanning electron microscopy (SEM)[246] by fixing at 37°C for 30 min in 2.5% glutaraldehyde (Sigma) in 0.1M cacodylate buffer containing 0.1M sucrose (Sigma) followed by two rinses in the buffer. The cells were then dehydrated with increasing concentrations (25, 50, 75 and 100%) of acetone in distilled water, by incubating for 10 min in 2 changes of each concentration. The fixed cells were taken to the Western General Hospital,

Edinburgh where they were Critically Point Dried using liquid CO_2 by the staff of the Electron Microscopy Suite of the MRC Population Cytogenetics Unit, Edinburgh. The cells were mounted on stubs and coated with gold using a gold sputter coater (Nanotech, England) prior to examination on a scanning electron microscope (Cambridge Stereoscan 250 Mark II, Cambridge Instruments Ltd, England).

2.29 Preparation of Cells/Spores Following Challenge with Spores in vivo

C57Bl/6 mice which had received an intraperitoneal injection of 3% thioglycollate (0.5 ml) 4 days previously, were challenged intraperitoneally with 10^8 spores in 0.5 ml HBSS. After 1.5 h the peritoneal cavity was lavaged with 5 ml of HBSS; the lavaged cell population with attached or ingested spores was allowed to adhere to coverslips for one hour at 37°C and was then prepared for light and scanning electron microscopy.

2.30 Chemiluminescence

This assay is based on the finding that stimulated phagocytes generate light detected as chemiluminescence[247] following contact with surface activating agents. The chemiluminescent responses of PMN and mononuclear cells were amplified by the addition of 5-amino-2, 3-dihydro-1, 4-phthalazinediane (luminol, Sigma)[248], whilst that of macrophages was amplified by bis-n-methylacridinium nitrate (lucigenin, Sigma)[249]. Luminol was made up by dissolving 17.7 mg in a minimal volume of dimethyl sulphoxide (Sigma) and making up to a concentration of 4 x 10^{-5}M in HBSS. Aliquots of stock solution were stored at -70°C until

required. Lucigenin was prepared by dissolving 51.0 mg into 10 ml HBSS to give 10^{-3}M. Aliquots were stored at -70°C until required. Chemiluminescence was measured directly in mV in an automated 24 sample carousel LKB 1251 luminometer interfaced to an Apple IIe computer with disc storage. Phagocytic cells were prepared at 5 x 10^6/ml in cold HBSS and maintained on ice until required. Optimal spore:cell ratios for the assay systems were determined (Figs 2.15 a,b and 2.16) and the following concentrations used: PMN 10:1, monocytes 50:1, macrophages 100:1. Zymosan was used as a positive control. Standardisation experiments showed that a concentration of 5 mg gave good responses for both PMN and monocytes (Table 2.9). A volume of 200 µl of opsonised spores or zymosan was placed in polystyrene cuvettes (LKB) followed by 200 µl of the appropriate amplifier. Luminol for PMN and mononuclear cells and lucigenin for macrophages. Five hundred microlitres of cells were then added (2 x 10^6/ml) and the cuvettes immediately placed into the carousel of the luminometer and kept at 37°C with regular mixing. The chemiluminescent responses were measured in mV and automatically recorded at timed intervals until the chemiluminescence had peaked. All results were stored on disk for subsequent analysis. A typical chemiluminescent response of both PMN and mononuclear cells to zymosan is illustrated in Fig 2.17. As the number of samples which could be tested at any one time was governed by the availability of cells and the capacity of the machine it was decided that each treatment would be measured once only. This decision was based on the results of experiments which showed that the technique gave highly reproducible results within replicates. An example of the reproducibility of the measurements of chemiluminescent responses of zymosan stimulated mouse peritoneal

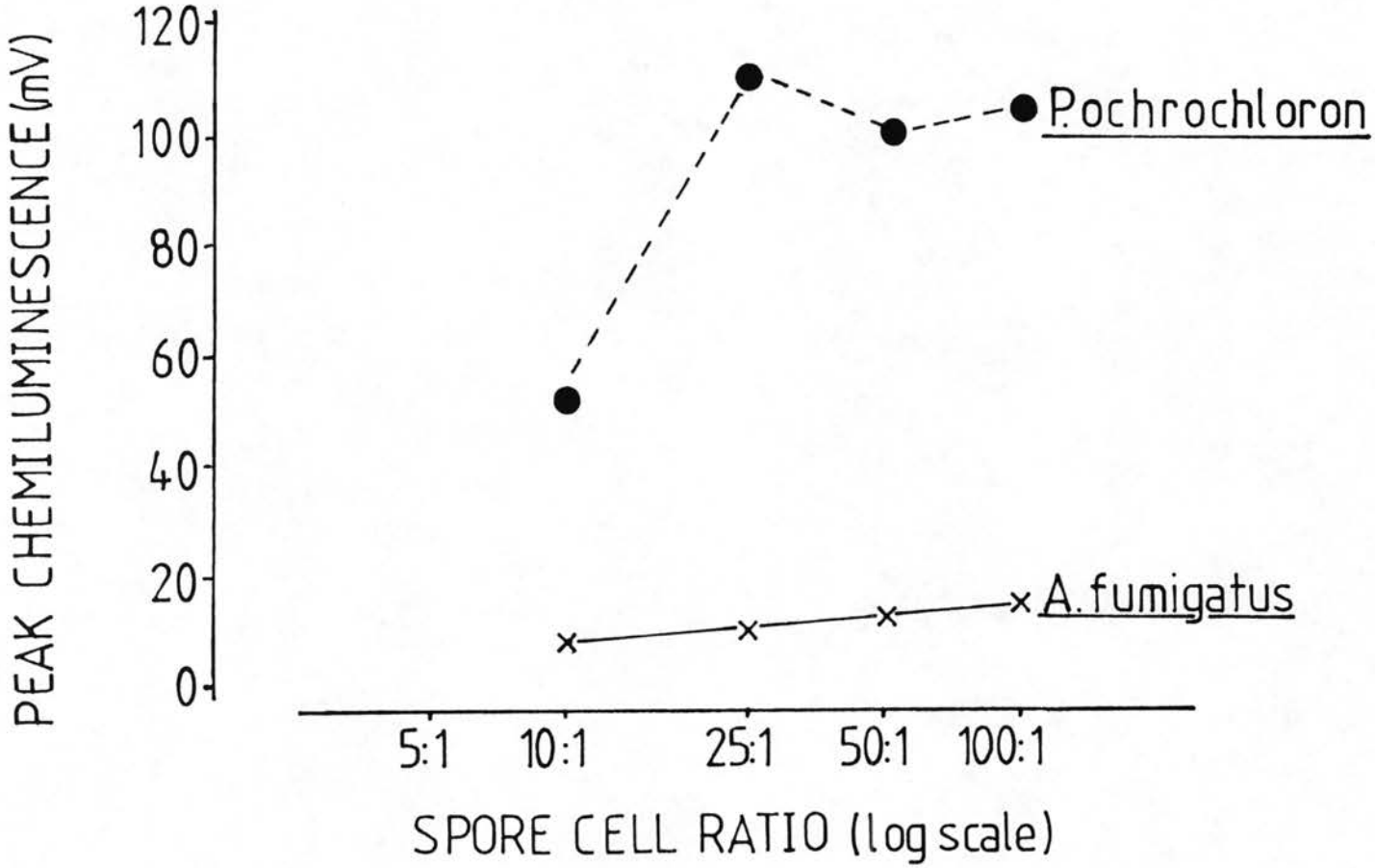


Figure 2.15a The effect of increasing spore:cell ratio (5:1 - 100:1) on the luminol amplified chemiluminescent responses of human monocytes towards spores of A. fumigatus and P. ochrochloron, opsonised in 5% human AB sera. Results expressed as peak chemiluminescence in mV.

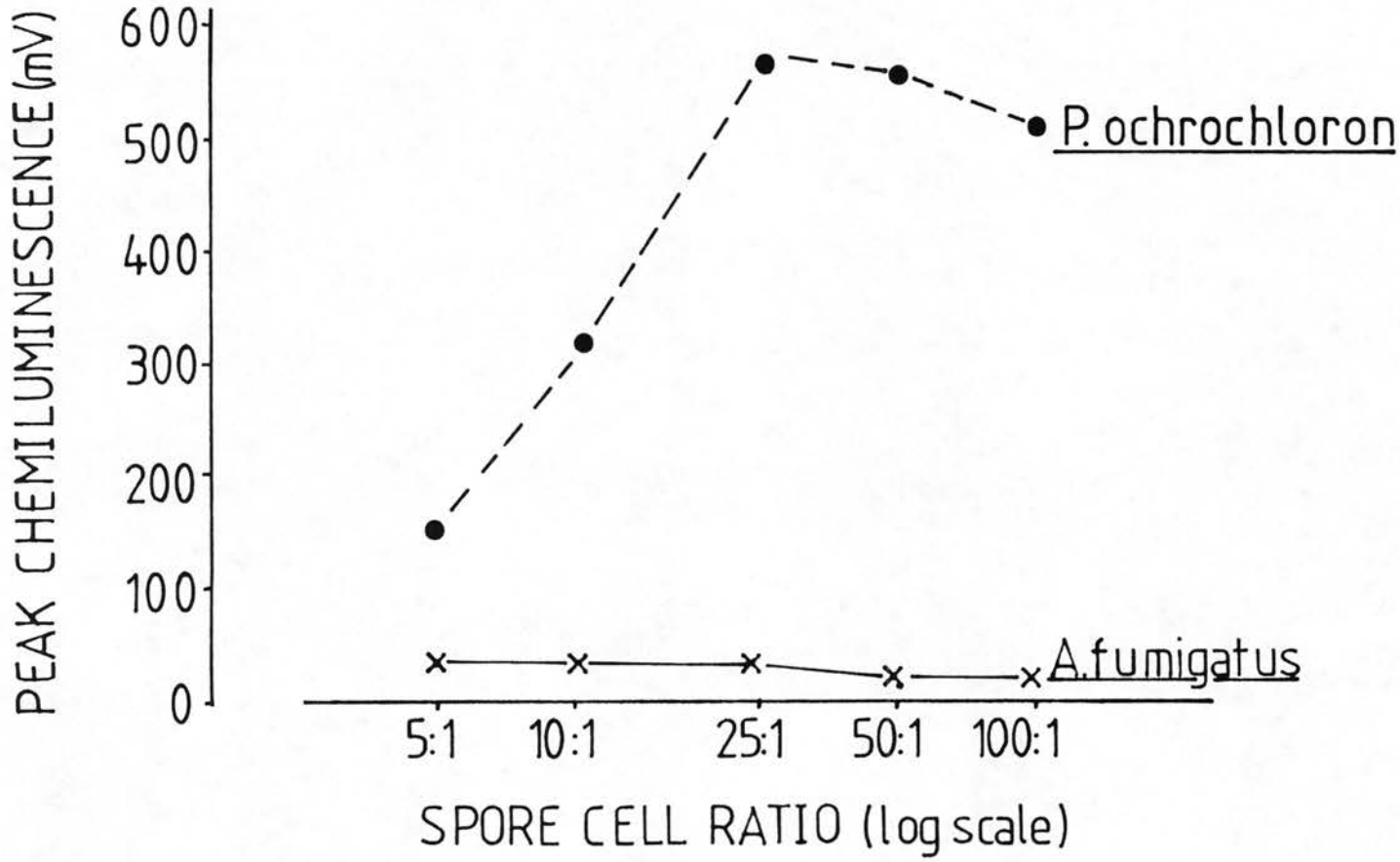


Figure 2.15b The effect of increasing spore:cell ratio (5:1 ~ 100:1) on the luminol amplified chemiluminescent responses of human PMN towards spores of A. fumigatus and P. ochrochloron, opsonised in 5% human AB sera. Results expressed as peak chemiluminescence in mV.

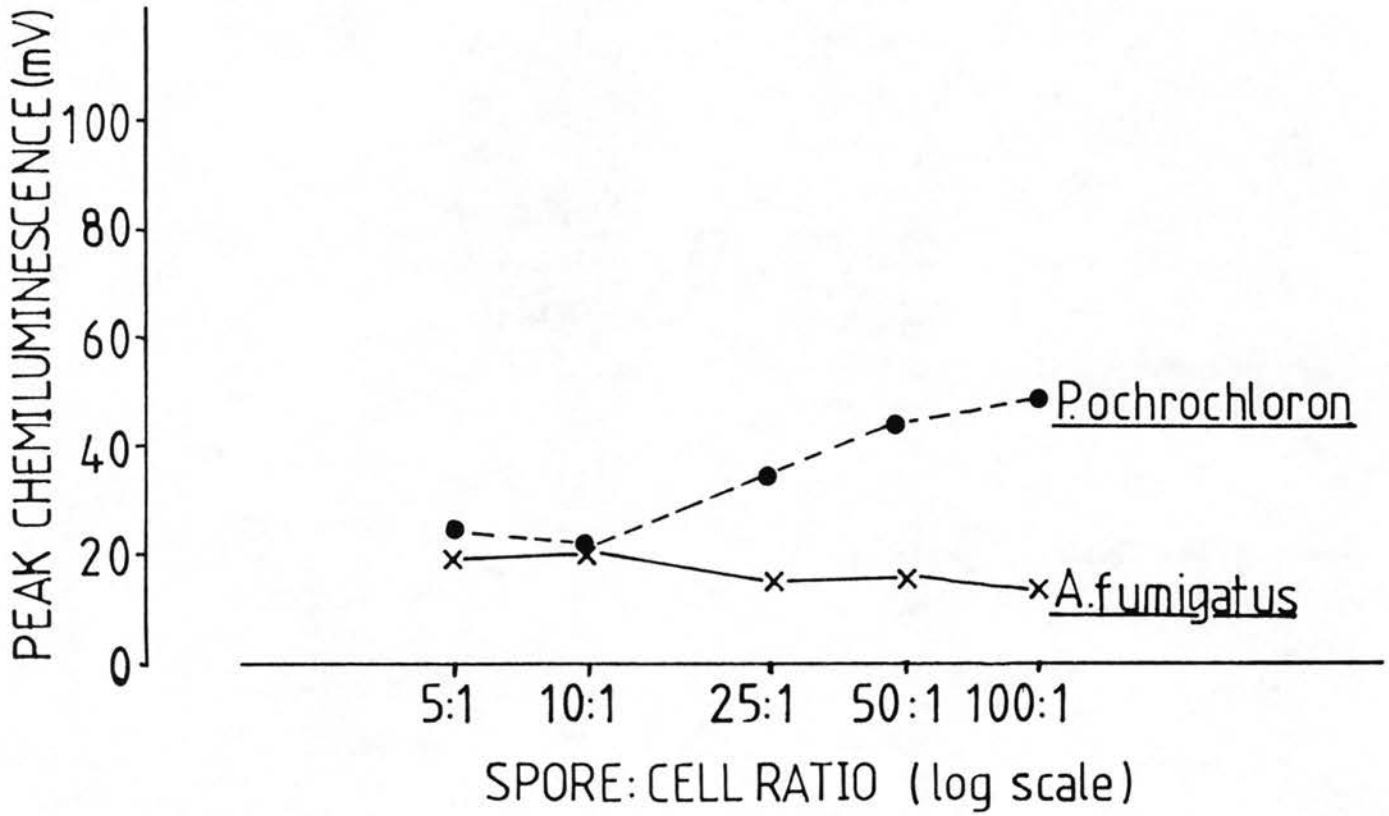


Figure 2.16 The effect of increasing spore:cell ratio (5:1 100:1) on the lucigenin amplified chemiluminescent responses of mouse peritoneal exudate cells towards spores of A. fumigatus and P. ochrochloron, opsonised in 5% autologous sera. Results expressed as peak chemiluminescence in mV.

TABLE 2.9 The effect of increasing concentrations of zymosan, from 0.5-20 mg, on the production of chemiluminescence by human monocytes and PMN

Zymosan* mg	PMN		Monocytes	
	Peak (mV)	Time (sec)	Peak (mV)	Time (sec)
0.5	554.9	1235	71.81	1495
2.5	770.4	845	100	1430
5.0	995.6	780	106	975
10.0	1103	650	109.1	650
20.0	1166	585	90.79	520

* Zymosan preopsonised in 5% human AB serum

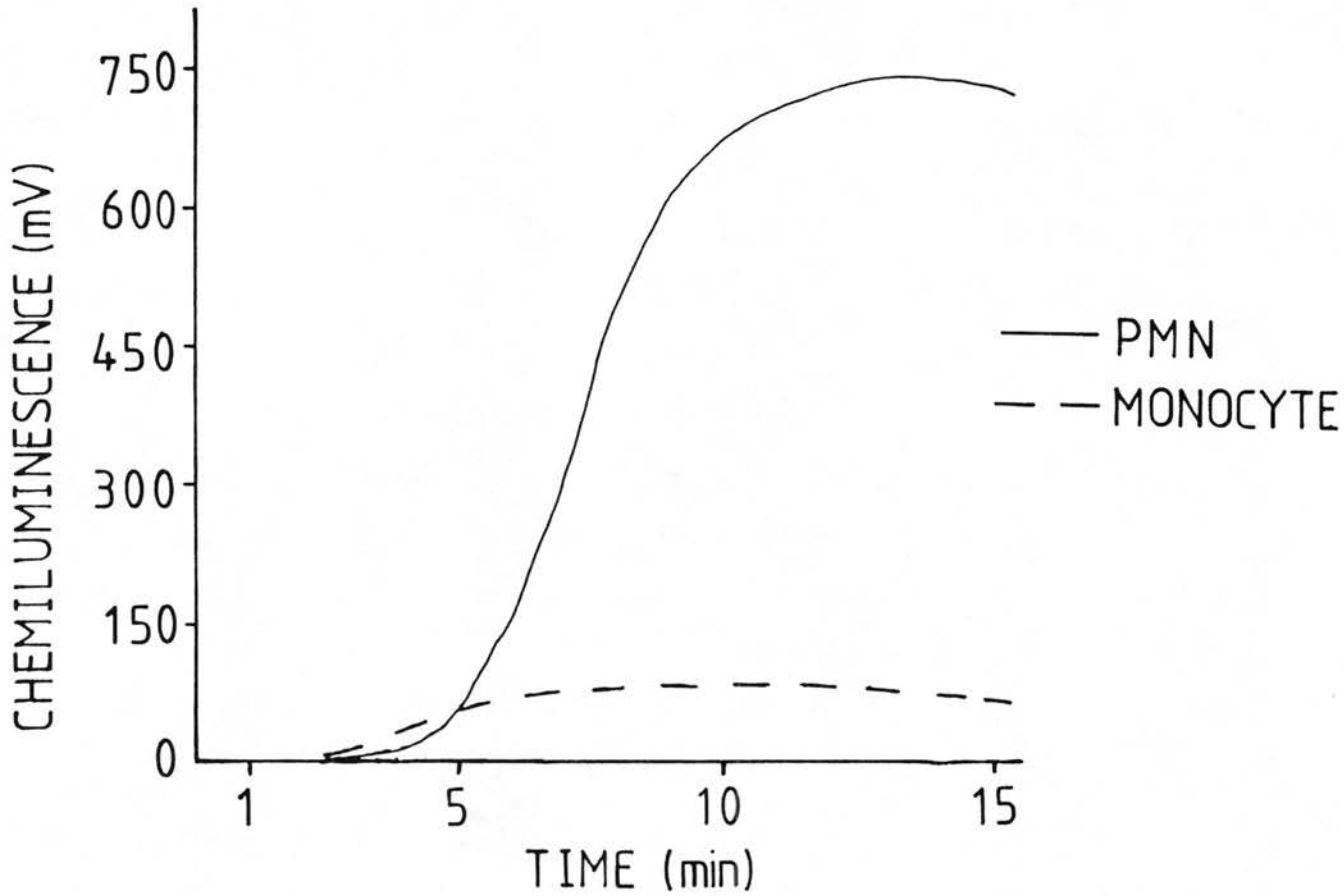


Figure 2.17 Typical profile of the luminol-amplified chemiluminescent responses of human PMN and monocytes (1 x 10^6 cells), towards zymosan (5 mg) monitored over a 15 min time period.

exudate cells are shown in Table 2.10. The coefficient of variation of this experiment was estimated at 1.5%.

2.31 Hydrogen Peroxide Assay

Hydrogen peroxide was measured according to the method of Pick and Keisari[250]. The assay is based on the horseradish peroxidase mediated oxidation of phenol red by hydrogen peroxide, which results in the formation of a compound demonstrating increased absorbance at 610 nm. Phenol red (Sigma) was made up to 0.028 M in distilled water (1 g/100 ml). This solution was kept at 4°C for up to 6 months. Horseradish peroxidase Type II salt-free powder (Sigma) was dissolved in 0.05 M potassium phosphate buffer pH 7.0 at a concentration of 5 mg/ml divided into one millilitre volumes and kept frozen at -70°C until required. Hydrogen peroxide was obtained as a 30% solution (Sigma) and 102 µl of this was diluted with 100 ml distilled water (0.01 M) just before use. Catalase was obtained as a purified powder from bovine liver (Sigma) and was diluted in 0.14 M sodium chloride just prior to use. The buffered phenol red solution (PRS) used in all assays contained 140 mM sodium chloride, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.28 mM phenol red and 50 ug/ml horseradish peroxidase.

The reaction mixture (1 ml) containing 5 x 10^5 phagocytic cells, phenol red solution and appropriate treatments, (for example, spores or spore diffusates) were added to 30 mm petri dishes (Sterilin). As an additional control, opsonised zymosan was added to the reaction mixture at a concentration of 1 mg/ml. All experiments on human cells

TABLE 2.10 Reproducibility of eight separate estimations of the chemiluminscent responses of lucigenin amplified mouse peritoneal exudate cells to zymosan opsonised in 5% autologous serum

Number of Estimations	Chemiluminscence Peak (mV)	Time (sec)
1	58.28	1170
2	57.64	1105
3	57.1	1105
4	55.86	1105
5	57.07	1105
6	56.43	1235
7	56.66	1040
8	58.18	1040

were set up in duplicate and whilst those on animal cells were carried out in triplicate; the dishes were incubated at 37°C for 2 h. After this time the supernatants were transferred into RT30 tubes which were spun at 400 *g* for 10 min at 4°C, to sediment cells/spores. The supernatants were then read at 610 nm using a spectrophotometer (Pye Unicam SP 400). A hydrogen peroxide standard curve was made by diluting the hydrogen peroxide stock solution to give concentrations of 10 µM to 10 mM. Ten microlitres of these hydrogen peroxide concentrations were added to RT30 tubes (Sterilin) containing 1 ml volumes of phenol red solution, to give a final hydrogen peroxide concentration of 0.1-100 µM. The standards were incubated at the same time as the assay. A typical standard curve is illustrated in Fig 2.18. The concentration of hydrogen peroxide in the samples was determined from the standard curve.

2.32 Superoxide Anion Assay

Superoxide anion was measured according to the method of Johnston[251] which relies on the reduction of cytochrome C, a reaction characterised by an increase in spectrophotometric absorbance of cytochrome C at a wavelength of 550 nm. The reduction of cytochrome C is not specific for superoxide anion and the required specificity is achieved by the use of superoxide dismutase, an enzyme for which superoxide anion is the only known substrate. This assay was run with and without superoxide dismutase and only the portion of the reduced cytochrome C which could be inhibited by superoxide dismutase was considered to reflect the nmol of superoxide anion released. The reaction mixture (total volume 1.5 ml) which contained 80 µmol cyto-

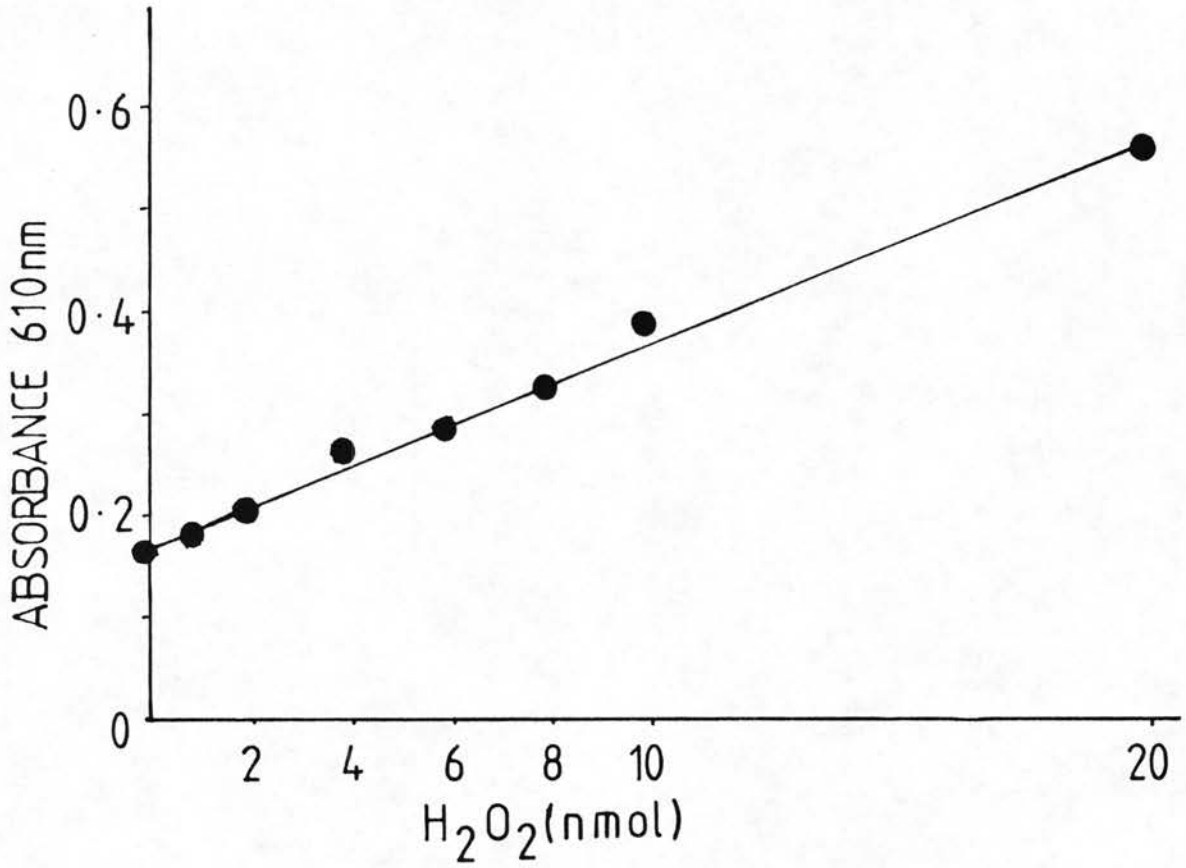


Figure 2.18 The effect of adding increasing concentrations of hydrogen peroxide (H_2O_2) to the spectrophotometric absorbance at 610 nm of the horseradish peroxidase-phenol red solution.

chrome C (Sigma) 2 mg/ml dextrose (Sigma), appropriate treatments (eg spores or spore diffusates) followed by 5 x 10^5 phagocytic cells were added to 30 mm Petri dishes. As an additional control, opsonised zymosan was added to the reaction mixture at a concentration of 1 mg/ml. To determine the portion of reduced cytochrome C that could be inhibited by superoxide dismutase identical dishes with added superoxide dismutase (25 ug/ml; Sigma) were prepared at the same time. The experiments were set up in either duplicate or triplicate and incubated at 37°C for 2 h. The supernatants were transferred into RT30 tubes and spun at 400 *g* for 10 min and 4°C. The peak absorbance at 550 nm, of the supernatant, was determined using the scan mode of the spectrophotometer (SP8/400 Pye Unicam). The portion of the reduced cytochrome C that could be inhibited by superoxide dismutase was then used to estimate the nmol of superoxide anion released.

2.33 Chemotaxis

In the chemotaxis experiments the basic procedure described by Snyderman[252] for blindwell chambers was followed except that PMN were used. The chemoattractant, zymosan activated serum was prepared by incubating 1 mg of zymosan with 1 ml of serum in a shaking water bath for 30 min at 37°C. The zymosan was spun out of the serum by centrifuging at 500 *g* for 15 min. The serum was then heat inactivated at 56°C for 30 min. Aliquots were stored at -70°C until required. The working solution used for the experiment was RPMI - 10 mM Hepes (Gibco) - 1% BSA.

A volume of 200 µl of chemoattractant was placed in the lower compartment of the blindwell chamber which was separated from the top compartment by a 3 µm nuclepore filter (Nuclepore Corporation Inc, Pleasanton, California USA). Prior to adding the cells (200 µl of 3 x 10^6 human PMN per ml) to the top compartment, the following solutions (200 µl) were added: (i) HBSS or (ii) A. fumigatus diffusate, or (iii) P. ochrochloron diffusate. For the experiments on random migration additional treatments were included. Instead of zymosan activated serum, 200 µl of HBSS or 200 µl of A. fumigatus diffusate were added to the lower compartment followed by 200 µl HBSS or 200 µl A. fumigatus diffusate to the top compartment. The chambers were incubated in a humidified incubator containing 5% CO_2 for 45 min at 37°C. After this time the filters were washed twice in PBS and stained in Diff Quick (Merz and Dade AG, Switzerland) and the number of migrated cells per five high power fields (magnification x 1000) were counted. All tests were carried out in duplicate. The basic procedure for this assay is illustrated in Fig 2.19.

2.34 Macrophage Spreading Assay

This assay was carried out according to the method of Donaldson et al[253]. Fifty µl of the following solutions was added to 6 x 22 mm glass coverslips:

i) control HBSS, or
ii) A. fumigatus diffusate, or
iii) P. ochrochloron diffusate.

To each of these coverslips were added 50 µl of 1 x 10^5 C. parvum

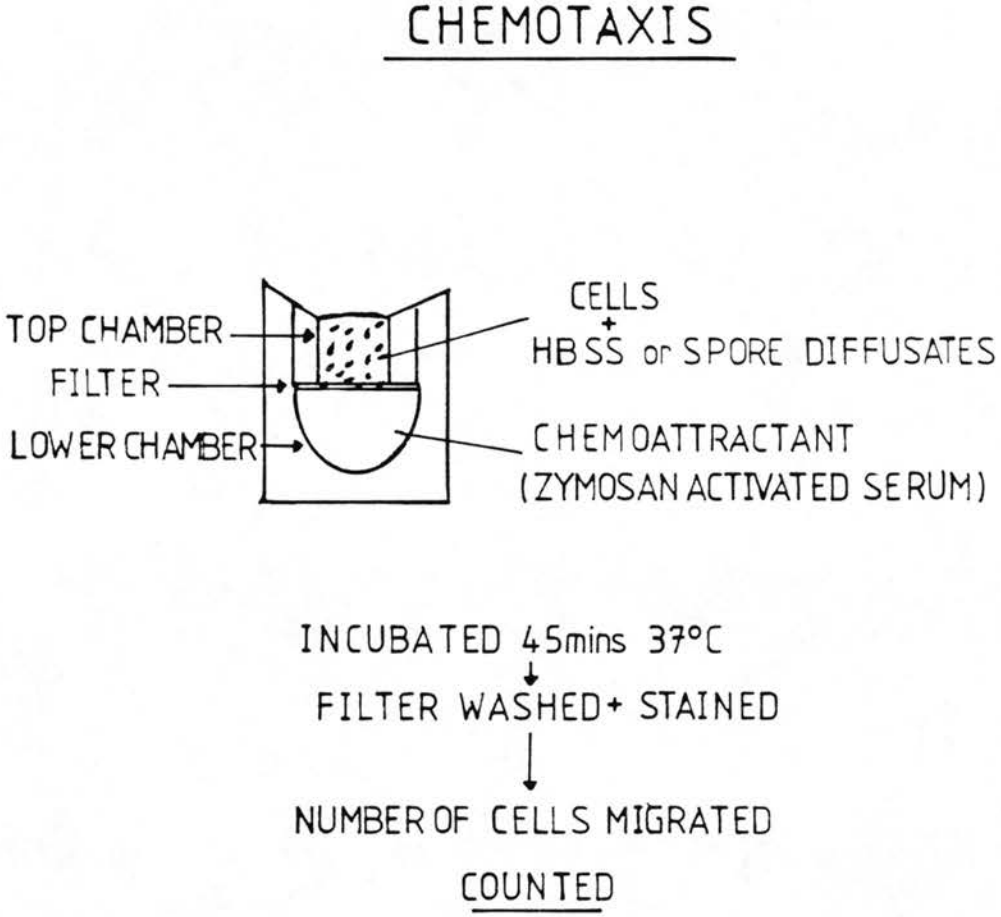


Figure 2.19 Illustration of the basic procedure used to estimate the effect of spore diffusates in the chemotaxis of human PMN towards zymosan activated serum using a blindwell chamber.

mouse peritoneal exudate cells in RPMI-1640 20% foetal calf serum which had been heat inactivated at 56°C for 30 min. After a one hour incubation at 37°C in 5% CO_2 the coverslips were rinsed gently twice in saline and stained in Diff Quick. When dry the coverslips were inverted and mounted on a microscope slide using DPX (BDH Chemicals Ltd, Poole, England). The greatest diameter of 200 cells was measured using a microcomputer-assisted digitising system (DDSI Graphic Information Systems Ltd, Scotland). With this system a pin-point of light on the cursor is superimposed on the image of the stained macrophage preparation using a camera lucida attachment to a Nikon FE optical microscope. Before measuring each assay the system was calibrated using a graduated graticule which enabled the cell measurements to be expressed directly in microns. All tests were carried out in triplicate. Typical size distributions of resting and activated (C. parvum elicited) peritoneal exudate cells are illustrated in Figs 2.20 a,b.

2.35 Techniques to Detect a Possible Scavenging Effect of Diffusates on Reactive Oxygen Intermediates

The following methods were used to test for a possible scavenging effect of diffusates on reactive oxygen intermediates.

2.35.1 Superoxide anion

A cell-free superoxide anion generation system generally based on the method described by Rosen and Klebanoff was used[254]. Approximately 6 nmol of superoxide anion was generated by incubating xanthine oxidase (20 µg/ml, Sigma) with acetaldehyde (40 nmol, Sigma) for 5 min at 37°C. To this superoxide anion generating system one millilitre of

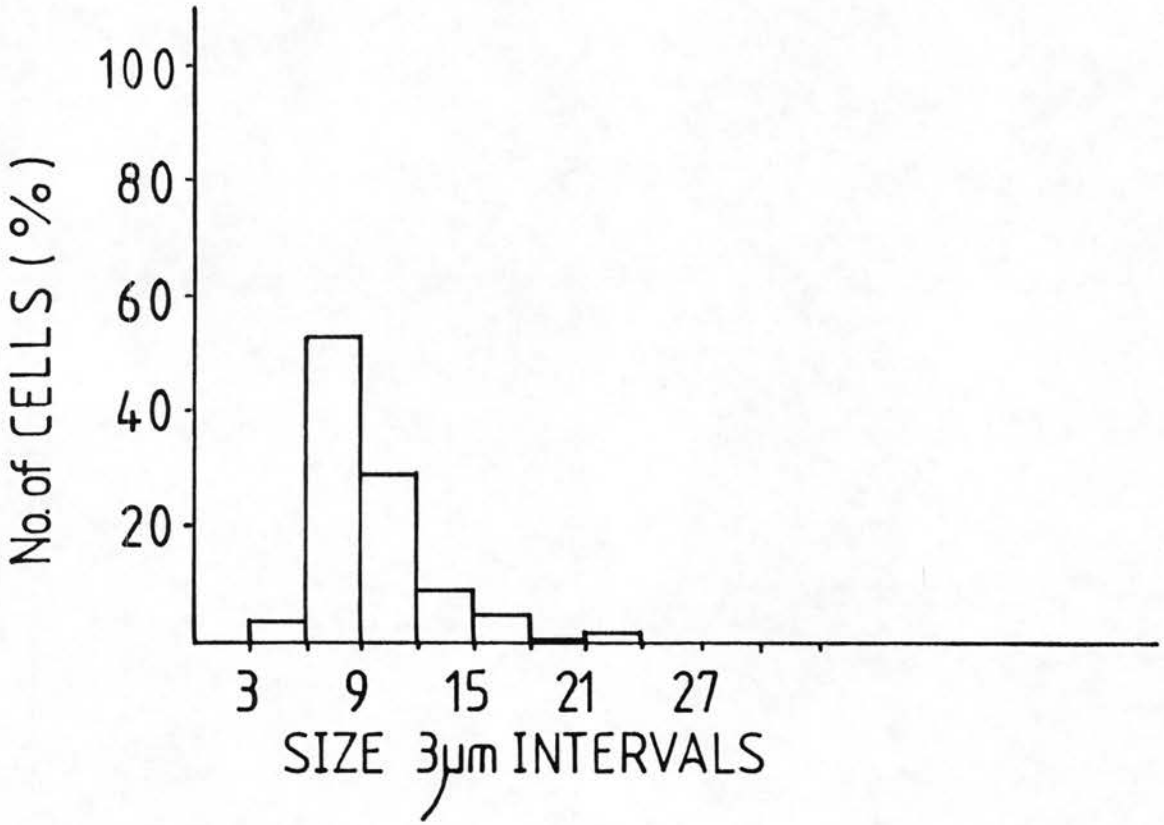


Figure 2.20a Typical size distributions of the diameter of naive (resting) mouse peritoneal exudate cells following incubation on glass in RPMI-10% foetal calf serum (heat inactivated) for 1 h at 37°C.

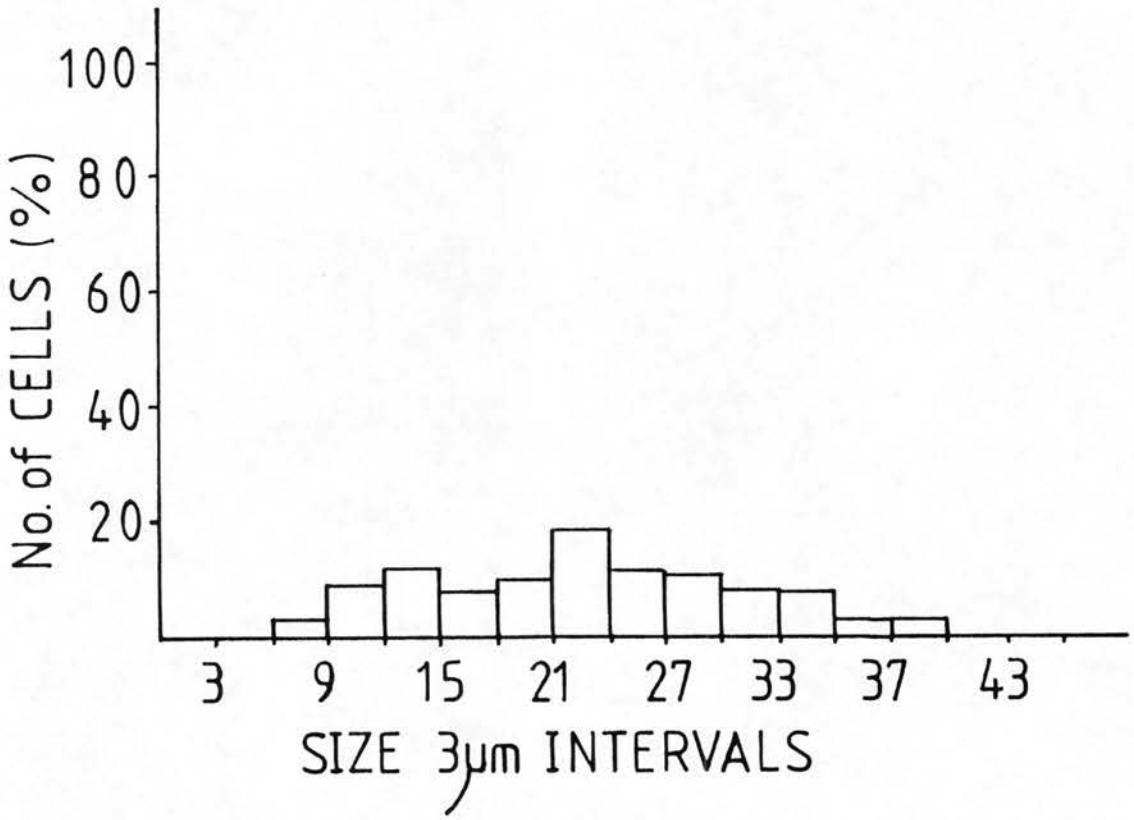


Figure 2.20b Typical size distributions of the diameter of C. parvum stimulated mouse peritoneal exudate cells following incubation on glass in RPMI-10% foetal calf serum (heat inactivated) for 1 h at 37°C

the following was added: (a) HBSS (control); (b) superoxide dismutase (25 ug) (c) diffusate (1:4 dilution). Each of the 3 treatments was set up in triplicate and incubated for 10 min at 37°C. The reaction mixture, used to detect superoxide anion (see section 2.32), was then added and after a further incubation for 10 min at 37°C the absorbance at 550 nm measured.

2.35.2 Hydrogen peroxide

Hydrogen peroxide (Sigma) was diluted to give concentrations ranging from 1-20 nmol. To the hydrogen peroxide 1 ml of the following was added: (a) HBSS (control); (b) catalase (Sigma) 100 ug/ml, an enzyme which catalyzes the degradation of hydrogen peroxide to oxygen and water, and (c) diffusate (1:4 dilution). Each of the 3 treatments was prepared in duplicate and incubated for 30 min at 37°C. The reaction mixture used to detect hydrogen peroxide (see section 2.31) was added to the tubes which were then incubated for a further 30 min at 37°C; the absorbance at 610 nm of the mixture was read.

2.36 Techniques to Determine Molecular Weight of Spore Diffusates

To determine whether the spore diffusate was of a low molecular weight the diffusate was dialysed against HBSS for 16 h at 4°C using dialysis tubing 8/32 ins. (The Scientific Instrument Centre Ltd, London, England). As the activity of the diffusate appeared to decrease on storage at 4°C, an aliquot of the diffusate was put into dialysis tubing and kept in a sealed tube for 16 h at 4°C, as a control. The treated diffusate was then tested in the superoxide anion and hydrogen peroxide assay systems. The diffusate was also put through Amicon

filters (Stonehouse, UK) with exclusion limits of >1,000 MW, >5,000 MW and >10,000 MW.

2.37 Techniques to Assess the Time taken for Release of Spore diffusate

In order to find out at what stage the diffusates were released into the supernatant, spores (10^8/ml) were added to HBSS. This suspension was divided into 3 aliquots, and the following treatments carried out:

(i) the spores were incubated for 2 min immediately removed and supernatants filtered; hereafter referred to as 2 min incubation or washings;

(ii) the washed spores were resuspended in HBSS and incubated for 3 h at 37°C before spinning them out obtain the supernatant; hereafter referred to as washed + 3 h incubation;

(iii) the spores were incubated in HBSS for 3 h and the supernatant removed and filtered = 3 h incubation.

The spore diffusates were then tested in the superoxide anion and hydrogen peroxide assay systems.

2.38 Assessment of Cell Viability Following Treatment with Spore Diffusates

2.38.1 Trypan blue exclusion

To determine if the spore diffusates were having a toxic effect on the cells, 5 x 10^5 cells were incubated for 2 h at 37°C in 1 ml of control HBSS or spore diffusates (1:4 dilution). After this time the percent-

age viability was determined by the trypan blue exclusion method.

2.38.2 ^{51}Chromium release assay for detection of cytotoxicity

Human lung macrophages were added to flat-bottomed microtitre plates at a concentration of 1 x 10^5 cells per well in RPMI-2% BSA. Ten microlitres of ^{51}Cr (100 KBq) was added to the wells and the cells left to become radio-labelled for 18 h at 37°C in 5% CO_2. The cells were washed x 3 in PBS and 200 µl of the following added: (i) HBSS - a measure of spontaneous release (ii) A. fumigatus diffusate diluted 1:2 in HBSS, (iii) A. fumigatus diffusate diluted 1:4 in HBSS. The cells were incubated for a further 3 h at 37°C in 5% CO_2. A sample of 100 ul of the supernatant was taken and counted in the gamma counter. A total measure of the amount of ^{51}Cr taken up by the cells was made by adding distilled water-0.1% triton-X to wells containing the cells and HBSS; the cell lysates were then counted in the gamma counter. Cytotoxicity was determined by comparing the counts in the supernatants of the cells treated with diffusates, with those counts obtained by the addition of HBSS alone (spontaneous release)[255].

2.39 Enzyme Treatment to Strip Spores from Cell Surfaces

In an attempt to quantify the number of intra and extracellular spores an enzyme stripping technique was adopted. Opsonised spores of A. fumigatus were incubated with mouse peritoneal exudate cells in either medium plus 5% autologous serum alone (see section 2.25) or containing known inhibitors of phagocytosis; cytochalasin B (25 µg/ml) and sodium azide (0.02%). After one hour incubation at 4°C or 37°C the cells/spores were washed to remove excess non-cell-associated

spores and the cells treated with pronase (0.15%), an enzyme which is known to strip cell receptors and, therefore, the attached spores. The cells were then disrupted to release the remaining (ingested) spores (see section 2.27). The spores removed by stripping and obtained by breaking up the cells were then counted (see section 2.25).

2.40 Statistical Analyses

The majority of the statistical analyses was carried out by Mr W McLaren and Miss H P R Collins of the Statistical Branch at the IOM. The type of statistical analyses used depended on the nature of the experiments. Most of the work for the patient study was carried out on one occasion only. An analyses of co-variance was used to estimate the possible differences in the phagocytosis/killing assays. For standardisation work using the animals each experiment was carried out in triplicate and performed on 2-5 separate occasions. For individual experiments a different group of animals and spores or spore diffusates prepared from fresh cultures was used. Therefore, day to day variation in spontaneous release of ROI, killing of fungal spores and strength of diffusate did occur. The statistical analyses were designed to take into account this variation between experiments and treatments so that the analyses of treatment effect would be independent of inter-experimental variation. Initially the raw data were subjected to analysis of variance using a randomised block design; each day/experiment constituted a block. Student's-t tests were used to compare treatment means using the residual error obtained from the analysis of variance[256].

3. RESULTS

The mechanisms involved in the interactions of phagocytic cells with fungal spores were primarily assessed by measuring the processes of phagocytosis, killing and reactive oxygen intermediate production. The results of the experiments are presented in the order in which the research evolved. I thought this would give the reader a clearer understanding of the rationale behind the development and, therefore, the reasons for the experimental sequence. These general results are followed by the results of comparisons of fungal handling by phagocytic cells from asthmatic patients sensitised and non-sensitised to *A. fumigatus* and non-asthmatic control subjects.

3.1 Morphological Examination of the Interaction between Phagocytic Cells and Fungal Spores

This work began by examining morphologically what happens when fungal spores are added to phagocytic cells. The ability of phagocytic cells to bind fungal spores was assessed by estimating the number of spores which become cell-associated following incubation *in vitro* for one hour at 37^{o}C. Following these initial estimations of cell-association the next step was to distinguish between attached and ingested spores using Nomarski differential interference contrast optics, scanning electron microscopy and also enzyme stripping techniques.

3.1.1. Association of fungal spores with phagocytic cells

The results in Table 3.1 show that most (>77%) of the opsonised spores of both A. fumigatus and P. ochrochloron became cell-associated with both human monocytes and PMN after one hour incubation at 37°C. Similarly, approximately 65% of opsonised spores became cell-associated with mouse peritoneal exudate cells (Table 3.2). No significant differences were found between spore or cell type.

Examination by light microscopy of slide preparations of the cell-spore interactions also confirmed that a substantial number of spores had become cell-associated (Figs 3.1 a-d). It was not possible, however, to distinguish between attached and ingested spores.

Adherent cultures of thioglycollate elicited mouse peritoneal exudate cells challenged with spores of A. fumigatus were unable to fully inhibit spore germination. Phase contrast micrographs at two hours show that the spores had become cell-associated (Fig 3.2a). At nine hours, however, the spores had become swollen and germ tubes were beginning to emerge (Fig 3.2b) and by 12 h hyphal branching was evident (Fig 3.2c).

3.1.2 Discrimination between intracellular and extracellular spores

i) Examination of spores/cells using Nomarski Optics

Examination of the interaction of phagocytic cells from humans and mice with opsonised fungal spores after incubation for three hours at 37°C using Nomarski differential interference contrast optics revealed that a substantial number of spores appeared to be bound to

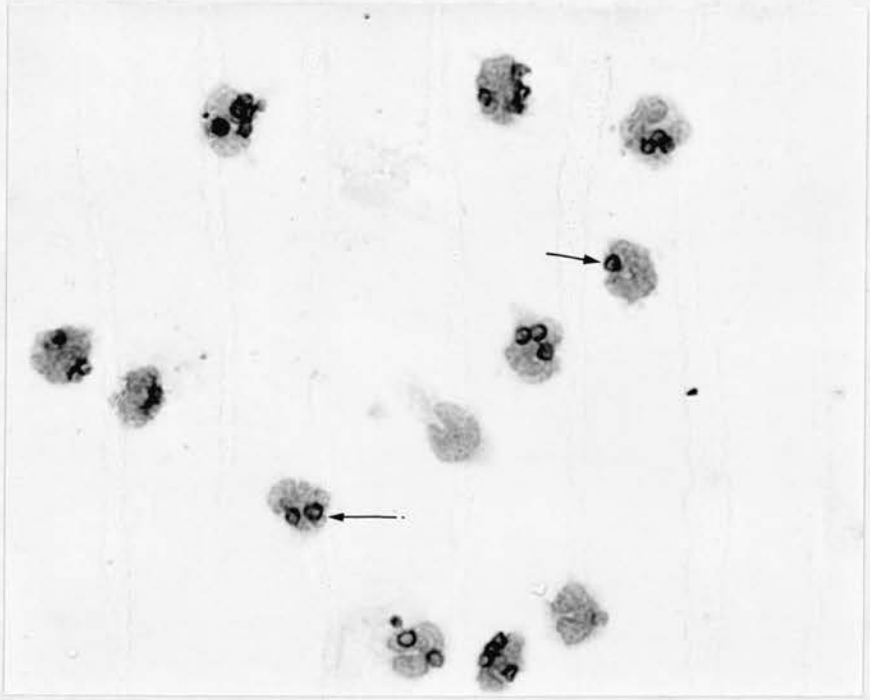
TABLE 3.1 The percentage of spores opsonised in autologous serum which became cell-associated with human monocytes and PMN after one hour *in vitro*

Fungal spore	Cell type	% of cell-associated spores
A. fumigatus	Monocyte	87.8 (3.2)*
A. fumigatus	PMN	80.4 (9.5)
P. ochrochloron	Monocyte	80.7 (4.7)
P. ochrochloron	PMN	77.5 (6.4)

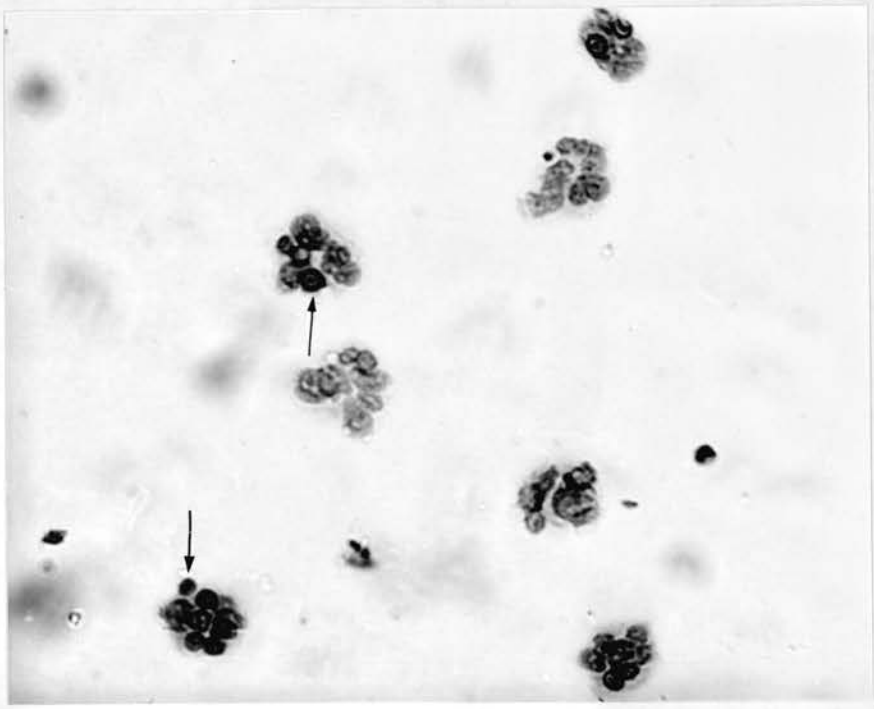
* Mean (SEM) of the results from 6 subjects

TABLE 3.2 The percentage of spores opsonised in autologous serum becoming cell-associated with thioglycollate elicited mouse peritoneal exudate cells after 1 h *in vitro*

Spore	No of Experiments	% cell-associated spores Mean (SEM)
A. fumigatus	7	65.6 (4.2)
P. ochrochloron	4	66.8 (4.1)

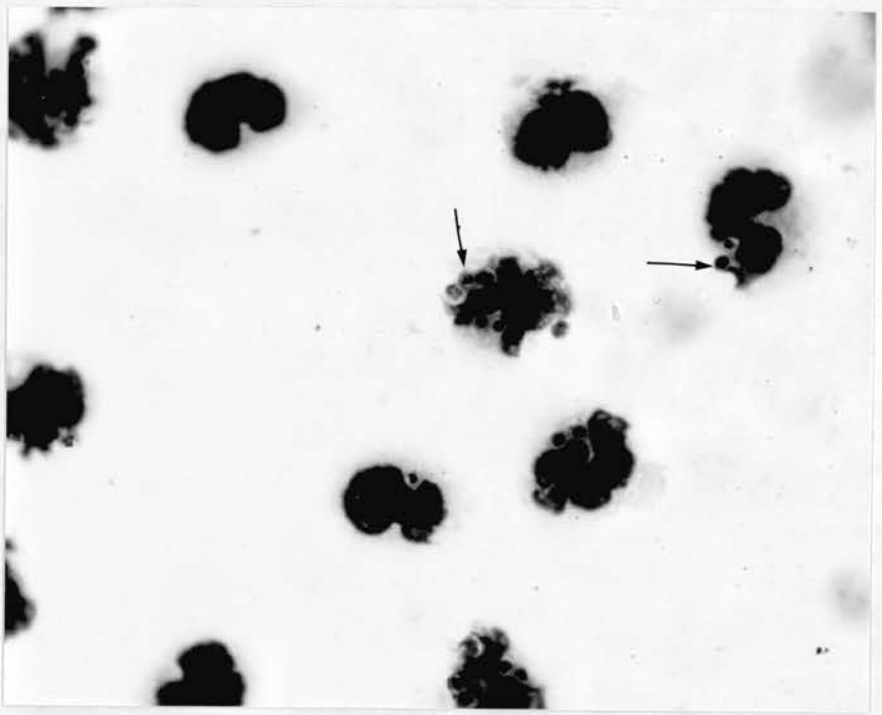


(a)

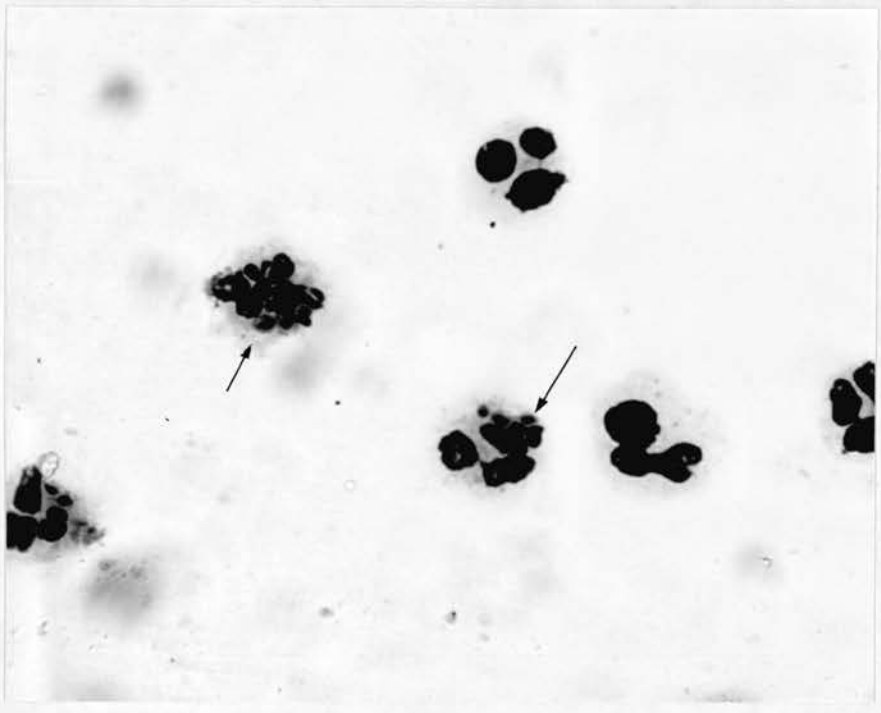


(b)

Figure 3.1 a-b Photomicrographs of the cell-association of human monocytes and PMN with spores of A. fumigatus (opsonised in 5% autologous serum) following incubation in vitro for 1 h at 37°C. (a) monocytes and A. fumigatus (b) PMN and A. fumigatus: spores indicated with arrows. Light microscopy original magnification x 400.



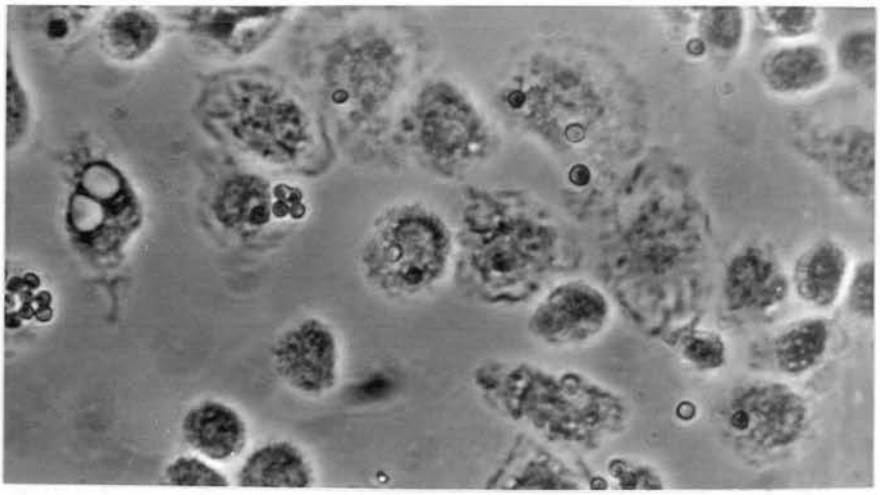
(c)



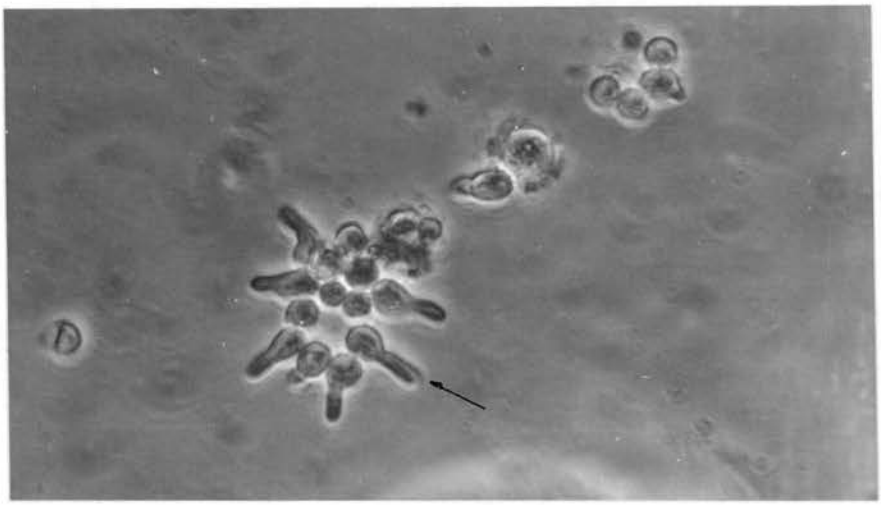
(d)

Figure 3.1 c-d Photomicrographs of the cell-association of human monocytes and PMN with spores of P. ochrochloron (opsonised in 5% autologous serum) following incubation in vitro for 1 h at 37°C (c) monocytes and P. ochrochloron (d) PMN and P. ochrochloron: spores indicated with arrows. Light microscopy original magnification x 400.

(a)



(b)



(c)

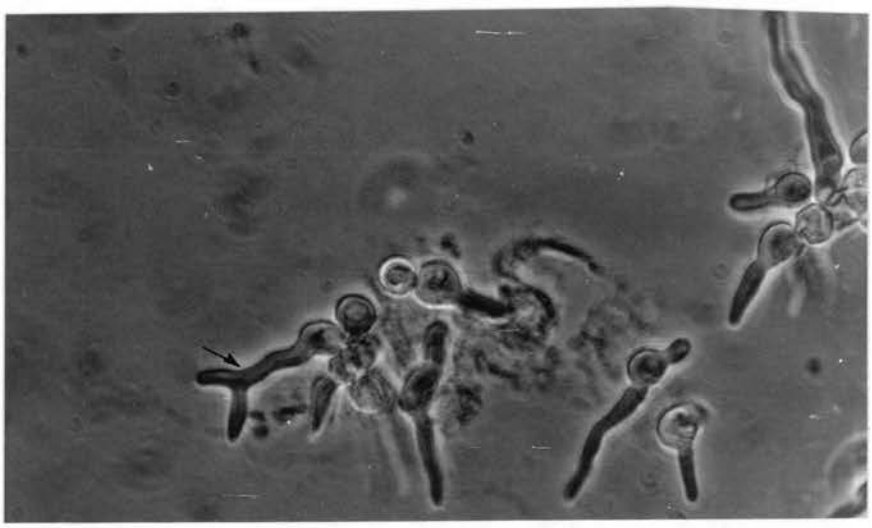


Figure 3.2 a-c Phase contrast micrographs of thioglycollate elicited mouse peritoneal exudate cells challenged with spores of A. fumigatus and incubated at 37°C for up to 12 h; (a) 2 h (b) 9 h germ tube indicated with an arrow (c) 12 h : original magnification x 400

the surface of the cell and not internalised. The spores which are seen as silhouettes against the cell surface are illustrated in Figs 3.3 a,b (with mouse macrophages), Figs 3.3 c,d (with human monocytes), Figs 3.3 e,f (with human PMN).

ii) Scanning electron microscopy of fungal spores with human phagocytic cells

The next stage was to examine the cell/spore interaction using scanning electron microscopy (SEM), which shows fungal spores as small rounded bodies closely associated with the phagocytic cell membrane. The degree to which the spore became cell-associated has been arbitrarily divided into three phases: (1) attached - spore lying on the cell surface; (2) partially ingested - the cytoplasm of the cell enclosing the spore yet not fully covering it; and (3) ingested - the outline of the spore being seen under the cytoplasm of the cell.

Using SEM a substantial number of spores of A. fumigatus were found to be attached to the surface of the human phagocytic cell and not internalised after incubation at 37°C for 1.5 h (Figs 3.4 a,b). Although spores of P. ochrochloron were present on the surface of the cell they appeared to be less numerous (Figs 3.4 c,d). Opsonisation of fungal spores in sera containing specific antibody did not appear to alter this finding.

iii) Examination of Fungal Spore Interactions with Mouse Phagocytic Cells using SEM

A mouse model was used to examine whether the finding (substantial number of extracellular spores) obtained by in vitro experimentation

(a)

(b)

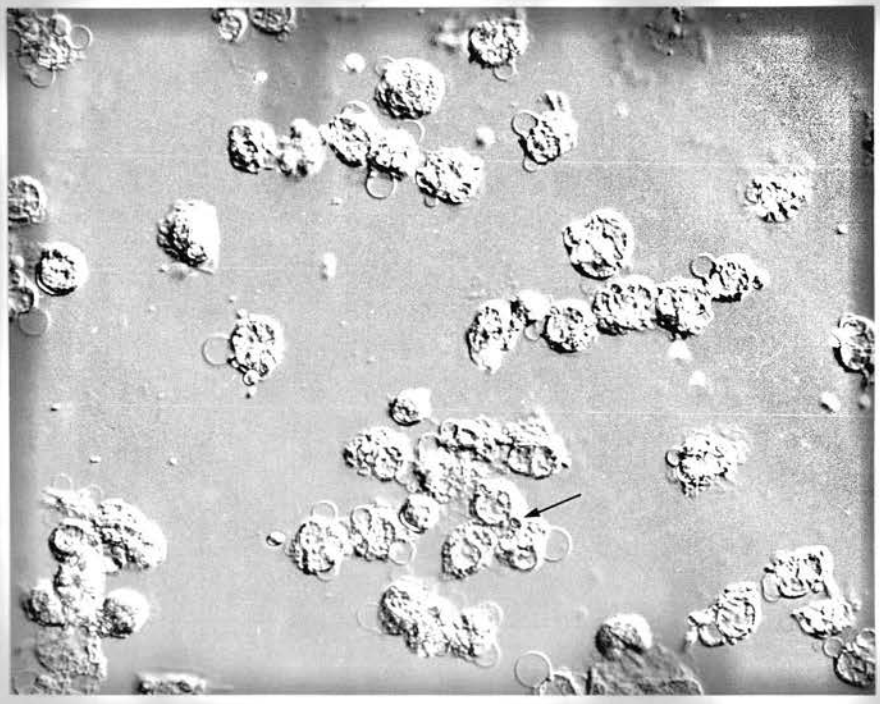
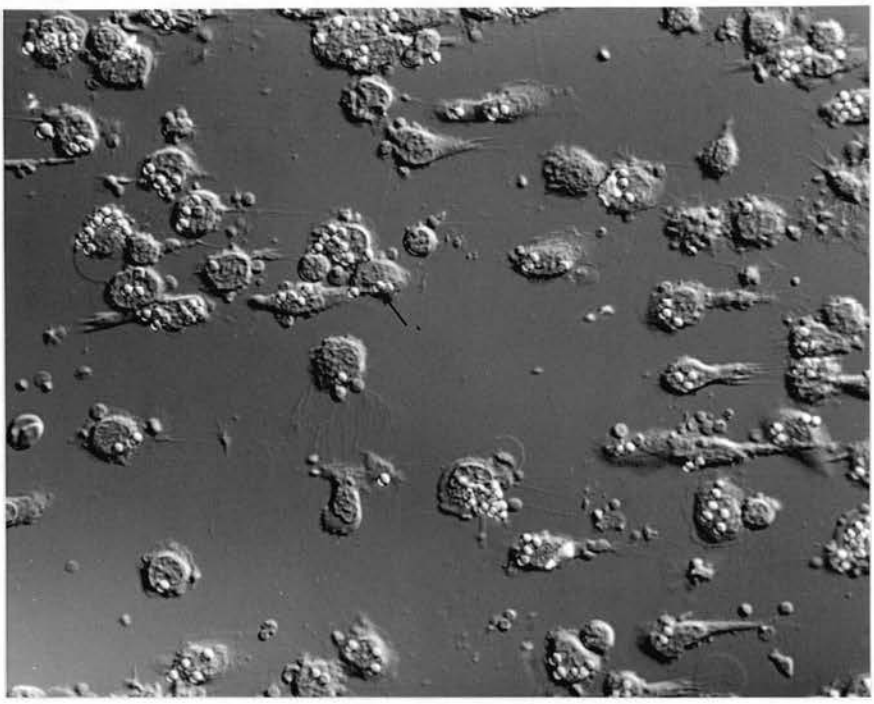


Figure 3.3 a-b Photomicrographs, using Nomarski differential interference contrast optics, of the cell-association of spores of A. fumigatus and P. ochrochloron opsonised in autologous serum with mouse peritoneal exudate cells following incubation at 37°C for 3 h; (a) A. fumigatus (b) P. ochrochloron, spores are indicated with arrows : original magnification x 400

(c)



(d)

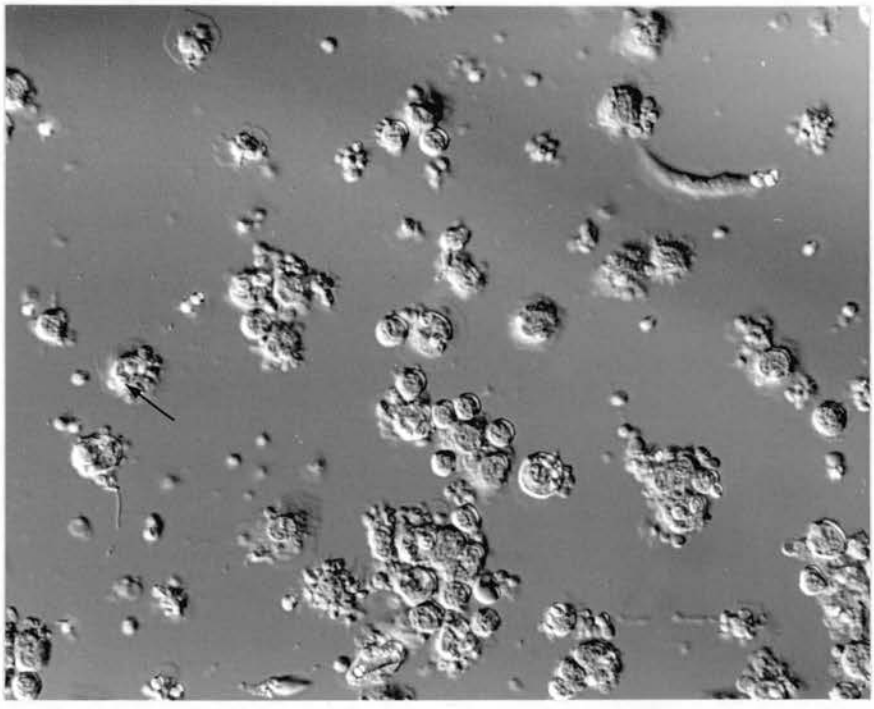
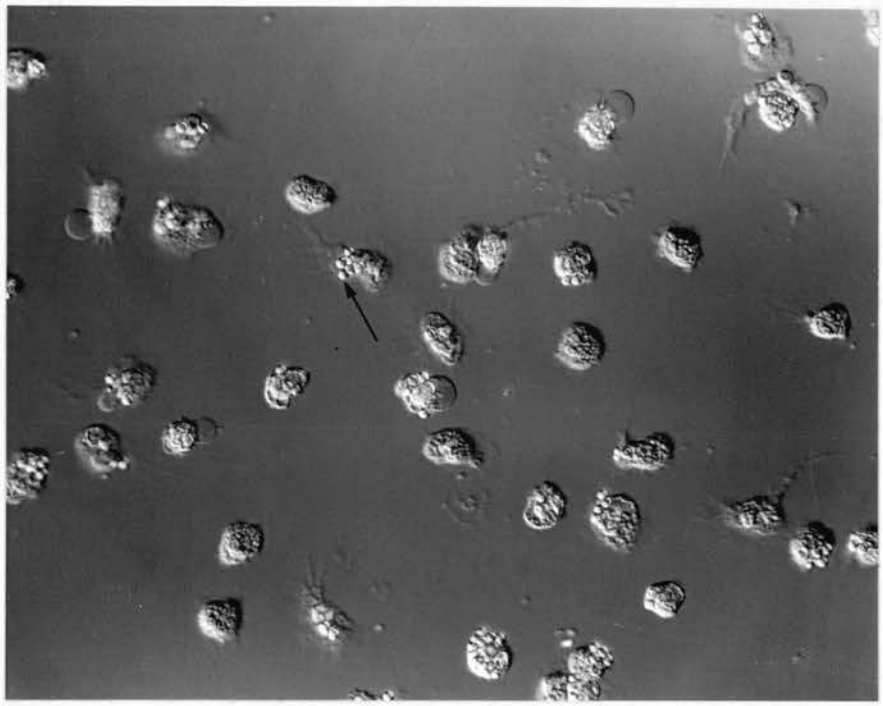


Figure 3.3 c-d Photomicrographs using Nomarski differential interference contrast optics of the cell-association of spores of A. fumigatus and P. ochrochloron opsonised in autologous serum, with human monocytes following incubation at 37°C for 3 h; (c) A. fumigatus (d) P. ochrochloron spores indicated with arrows; original magnification x 250.

(e)



(f)

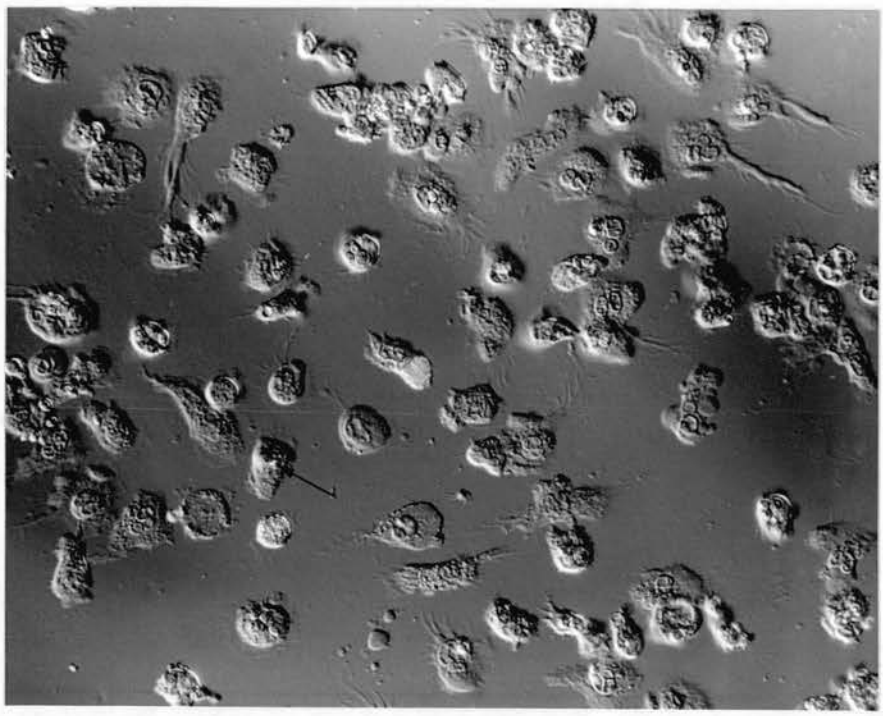
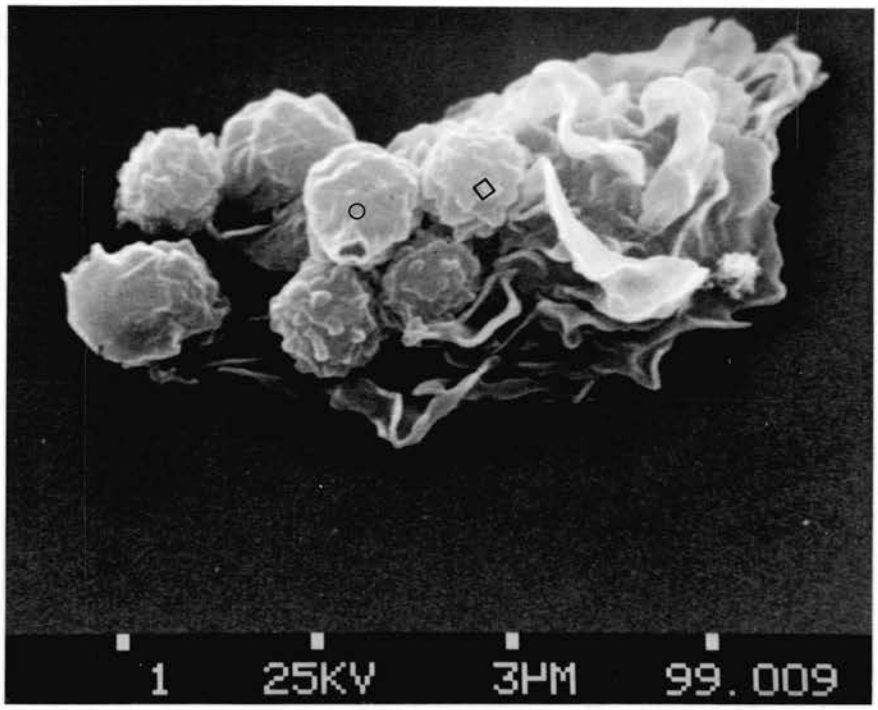


Figure 3.3 e-f Photomicrographs using Nomarksi differential interference contrast optics of the cell-association of spores of A. fumigatus and P. ochrochloron, opsonised in autologous serum, with human PMN following incubation at 37°C for 3 h; (e) A. fumigatus (f) P. ochrochloron spores indicated with arrows; original magnification x 250

(a)



(b)

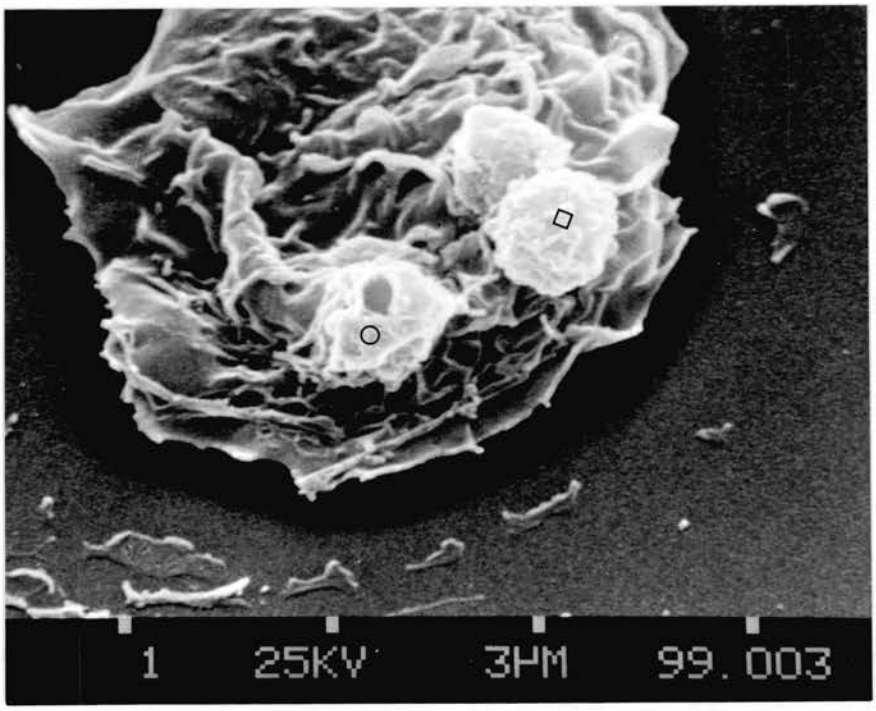
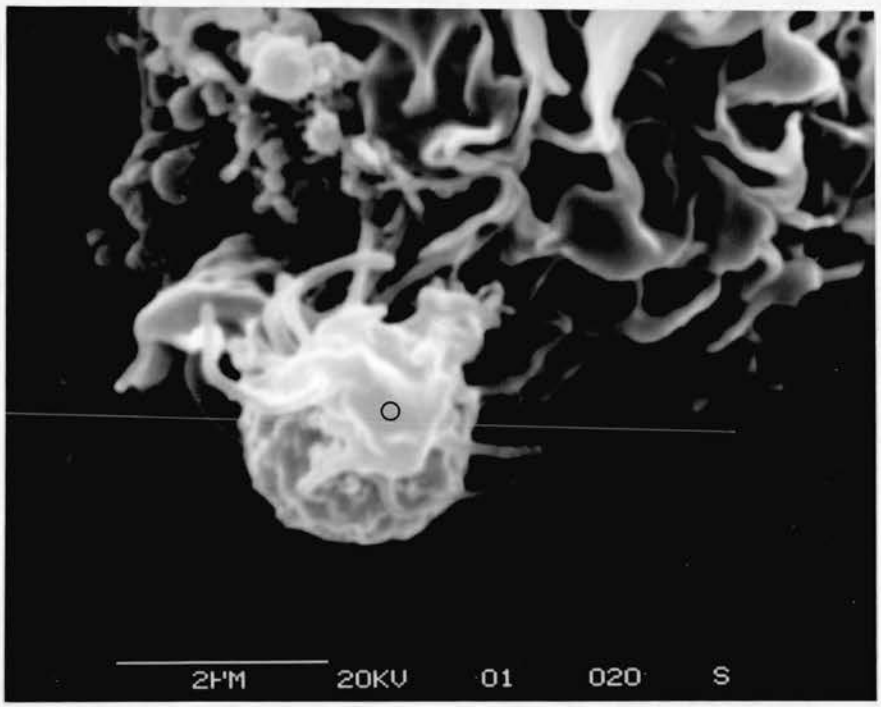


Figure 3.4 a-b Scanning electron micrographs of the interaction of spores of A. fumigatus, opsonised in autologous serum, following incubation in vitro for 1.5 h at 37°C: (a) a human monocyte (b) a human PMN; spores attached (□) partially ingested (○)

(c)



(d)

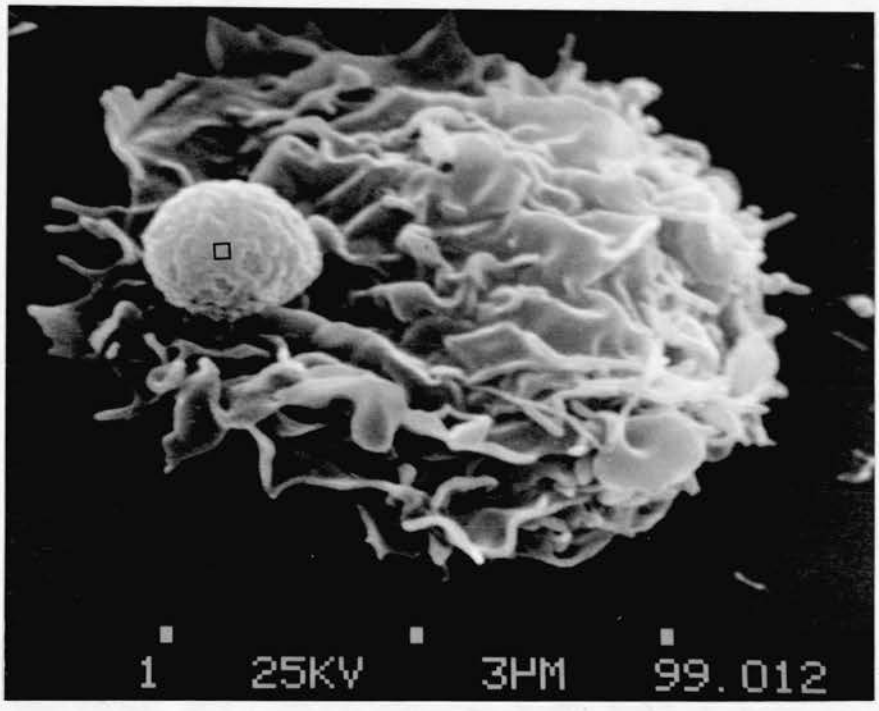


Figure 3.4 c-d Scanning electron micrographs of the interaction of spores of P. ochrochloron, opsonised in autologous serum, following incubation in vitro for 1.5 h at 37°C: (c) a human monocyte (d) a human PMN; spores attached (□) partially ingested (○)

could be repeated *in vivo*. Examination of the interaction of opsonised fungal spores with thioglycollate elicited mouse peritoneal exudate cells (PEC) *in vitro* gave results similar to those obtained with human phagocytes (Figs 3.5 a-b). The *in vivo* experiments, in which spores were injected into the peritoneal cavity of the mouse followed by lavage 1.5 h later, gave similar findings. A substantial number of *A. fumigatus* spores were seen to be bound to the surface of the cells *in vivo*, while spores of *P. ochrochloron* were becoming ingested (Figs 3.6 a-d).

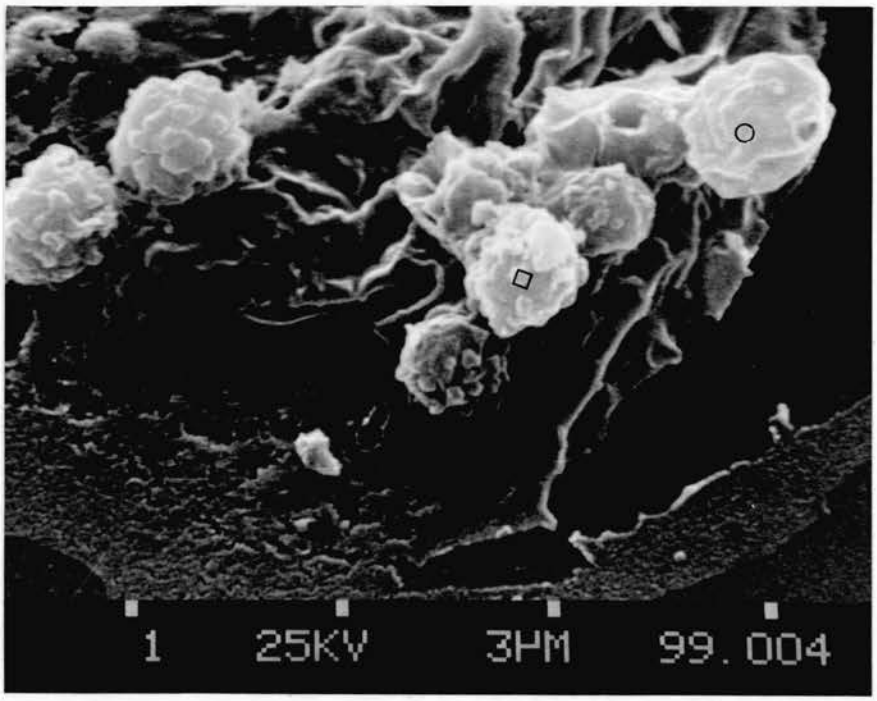
(iv) Quantification of the number of spores ingested using enzyme stripping techniques

Preliminary attempts, using enzyme stripping techniques to quantify the number of intra and extracellular spores were unsuccessful. Treatment with pronase dislodged substantially more spores from the phagocyte cell surface when compared with treatment by medium alone (Table 3.3). However, only 44% of the spores, which had become cell-associated by prior incubation at 4°C were dislodged, by pronase treatment even though the majority of these spores would have been surface bound and not internalized.

Summary

The results of all these experiments suggest that although *A. fumigatus* spores become cell-associated, the majority remain on the surface of the cell. However this was based on qualitative as opposed to quantitative data.

(a)



(b)

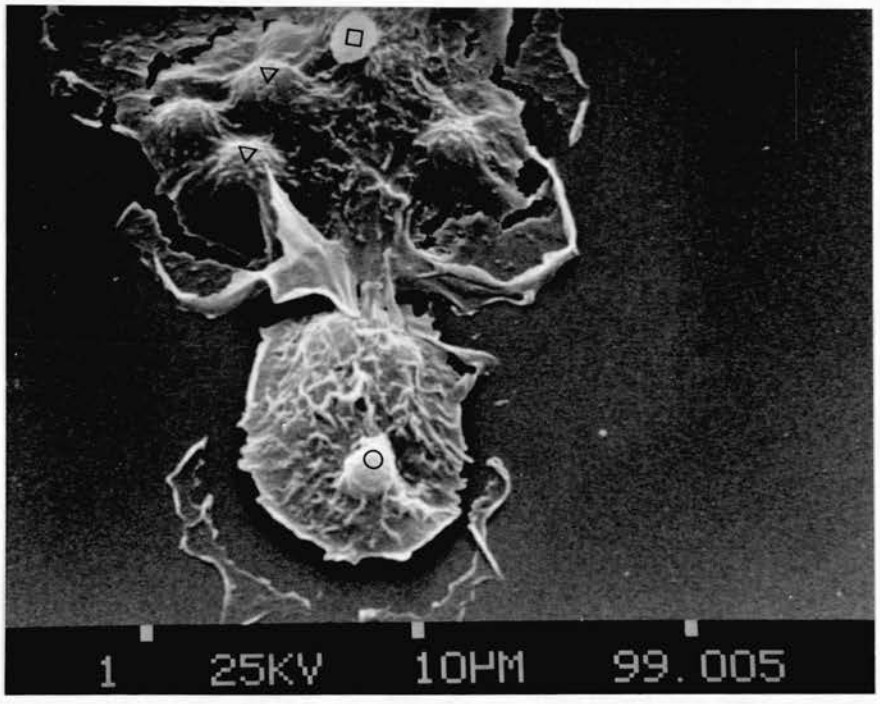
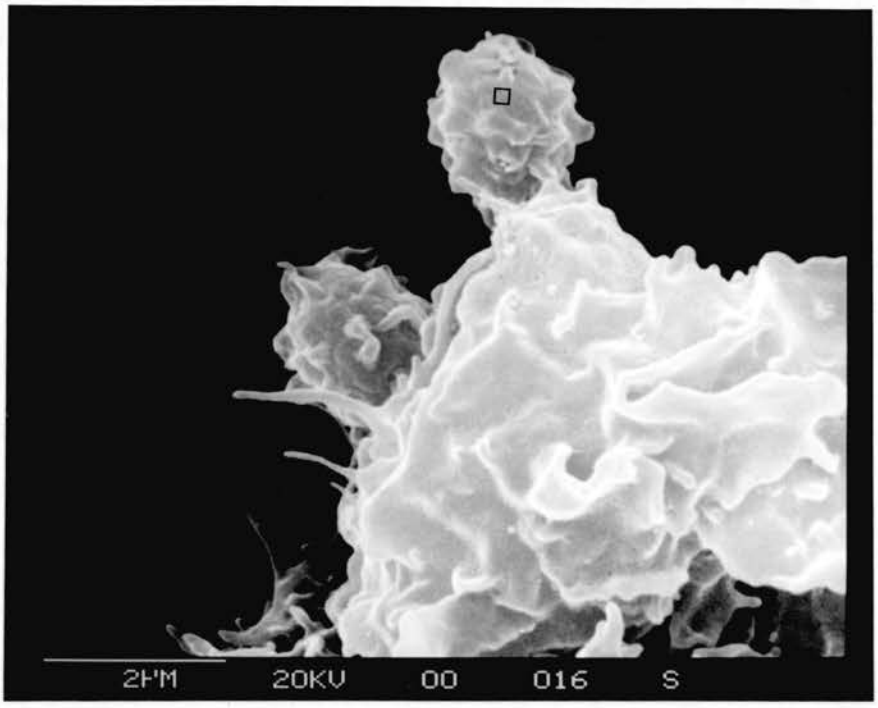


Figure 3.5 a-b Scanning electron micrographs of the cell association of fungal spores following incubation in vitro for 1.5 h of mouse peritoneal exudate cells and opsonised (a) spores of A. fumigatus (b) spores of P. ochrochloron. Spores : attached (□) partially ingested (○) fully ingested (▽).

(a)



(b)

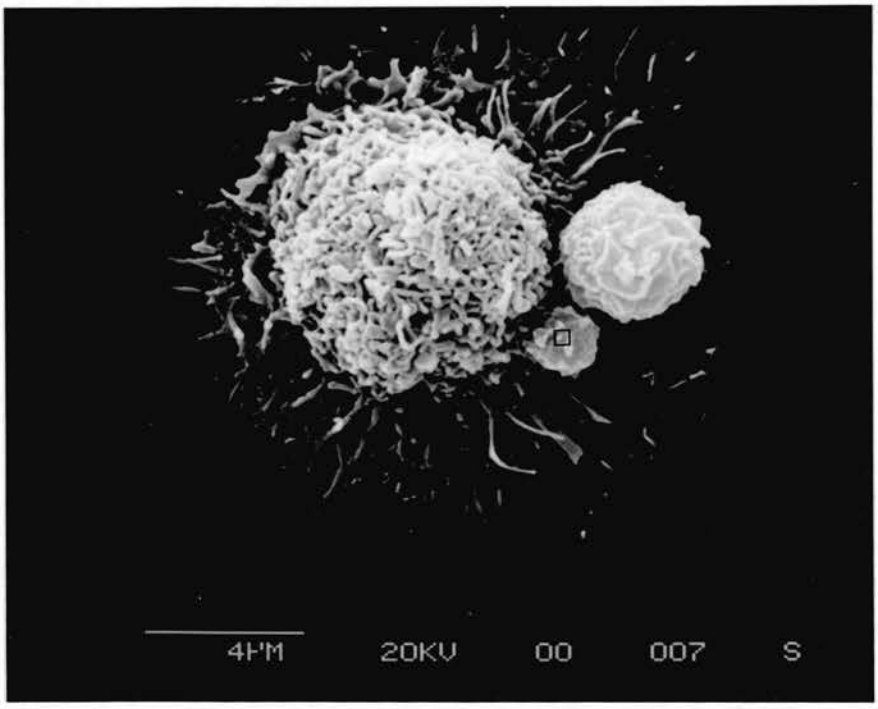
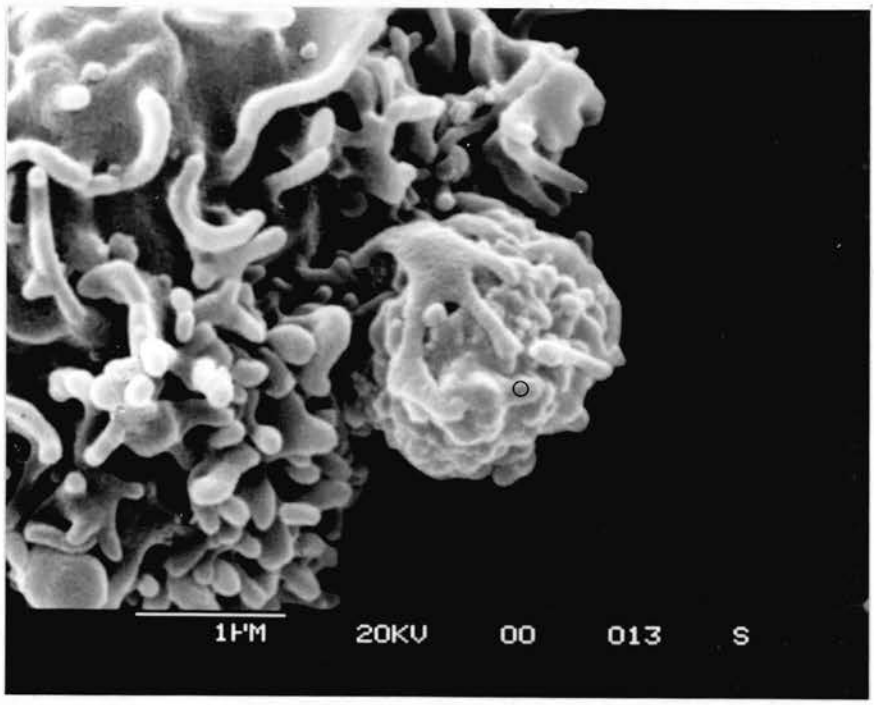


Figure 3.6 a-b Scanning electron micrographs of the cell-association of fungal spores following the interaction in vivo for 1.5 h of mouse peritoneal exudate cells and spores of A. fumigatus. Spores:attached (□).

(c)



(d)

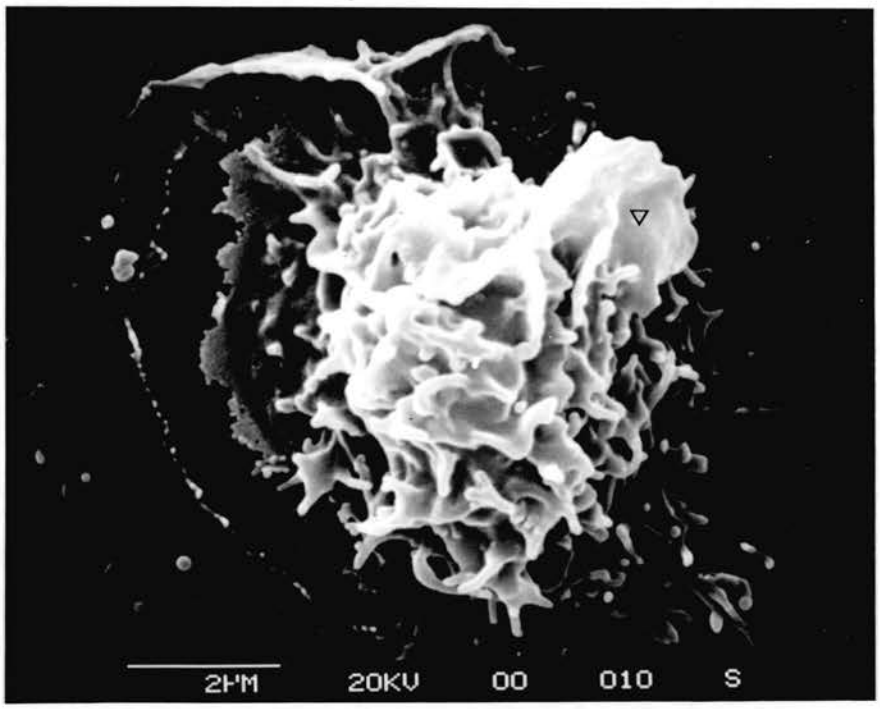


Figure 3.6 c-d Scanning electron micrographs of the cell-association of fungal spores following the interaction in vivo for 1.5 h of mouse peritoneal exudate cells and spores of P. ochrochloron. Spores : attached (□) partially ingested (○) fully ingested (▽).

TABLE 3.3 The percentage of cell-associated spores of A fumigatus becoming dislodged from mouse peritoneal exudate cells following treatment of spore/cell preparations with either medium (control) or pronase (enzyme). Prior to treatment the spores/cells were incubated for 1 h at either 4°C or 37°C

Incubation Temperature	Cell Treatment	
	Medium	Pronase
4°C	13*	44
37°C	9	40

* Percentage of spores becoming dislodged

3.2 Chemiluminescent Responses of Phagocytic Cells to Fungal Spores

Chemiluminescence is a measure of the light emited by phagocytic cells and is proportional to 'respiratory burst' activity. It arises from the production of high energy compounds intimately involved in the microbicidal activity of the cell.

A standardised chemiluminescence technique was used to answer the following questions:

(i) Do the chemiluminescent responses of human or murine phagocytic cells from A. fumigatus spores of differ from those to P. ochrochloron?

(ii) Does the magnitude of the monocyte 'respiratory burst' in response to fungal spores differ from that given by PMN?

(iii) Are the chemiluminescent responses of phagocytic cells different when spores are opsonised in autologous sera, in untreated or heated inactivated sera or in standard AB serum?

The amount of chemiluminescence produced by phagocytic cells varies from person to person and from day to day. Therefore, some of the results were expressed as a percentage of the peak chemiluminescent response of the phagocytic cells to zymosan on that day, which was always included as a positive control.

3.2.1 Chemiluminescent responses of phagocytic cells to spores of A. fumigatus compared with P. ochrochloron

The chemiluminescent responses of thioglycollate elicited mouse peritoneal exudate cells to spores of A. fumigatus opsonised in autologous sera was significantly lower than that obtained in response to P. ochrochloron ($p<0.01$, Table 3.4).

Spores of A. fumigatus (opsonised in AB sera) elicited a significantly lower ($p<0.001$) chemiluminescent response from human PMN and monocytes when compared with P. ochrochloron (Table 3.5). A typical chemiluminescent profile of human PMN in response to challenge with zymosan spores of A. fumigatus and P. ochrochloron is illustrated in Fig 3.7. Further experiments using different strains of A. fumigatus gave similar results. The peak chemiluminescent responses elicited by six different strains of A. fumigatus isolated from the sputum of a patient with aspergilloma are shown in Table 3.6. The peak chemiluminescence to A. fumigatus spores was in all cases substantially lower than that elicited by either zymosan or spores of P. ochrochloron.

3.2.2 Comparison of the chemiluminescent responses of PMN and monocytes

The peak chemiluminescent responses of PMN to zymosan or to spores of A. fumigatus or P. ochrochloron were substantially higher than those elicited by monocytes (Table 3.7). However, when the results were normalised by adjusting to the percentage of peak response to zymosan; this finding is reversed with the chemiluminescent responses of PMN to both A. fumigatus and P. ochrochloron falling to approximately 63% of that elicited by monocytes (the analysis of

TABLE 3.4 Chemiluminescent responses of thioglycollate elicited mouse peritoneal exudate cells to fungal spores opsonised in autologous serum

Trigger	Peak chemiluminescence (mV)
HBSS[1]	13.17 (3.63)[2]
A. fumigatus	14.45 (5.2)
P. ochrochloron	46.28 (6.9)*

1 Hanks' balanced salt solution - measure of the spontaneous release

2 Mean (SD) of 3 experiments

* Significantly greater than HBSS $p<0.01$

TABLE 3.5 Chemiluminescent responses of human PMN and monocytes to spores of *A. fumigatus* and *P. ochrochloron* opsonised in AB sera

Cell	*A. fumigatus*	*P. ochrochloron*
	peak chemiluminescence (mV)	
Monocyte	20.99 (5.82)[1]	69.2 (24.9)*
PMN	34.39 (6.59)	182.5 (69.7)*

1 Mean (SEM) of samples from 5 control subjects

* Significantly greater than *A. fumigatus* $p<0.001$

TABLE 3.6 The peak chemiluminescent responses of human phagocytic cells to 6 different isolates of *A. fumigatus* opsonised in AB sera

Trigger	Fungal Strain	Monocytes Peak chemiluminescence (mV)	PMN Peak chemiluminescence (mV)
Zymosan	-	168.3[1]	482.0
P. ochrochloron	-	174.3	368.9
A. fumigatus	Normal[2]	29.28	24.79
"	1	24.31	24.26
"	2	22.69	14.50
"	3	15.36	13.48
"	4	13.57	16.32
"	5	49.54	82.54
"	6	14.00	14.05

1 Result of a representative experiment

2 Strain of *A. fumigatus* normally used in the study

All *A. fumigatus* strains elicited significantly lower responses than *P. ochrochloron* $p<0.001$

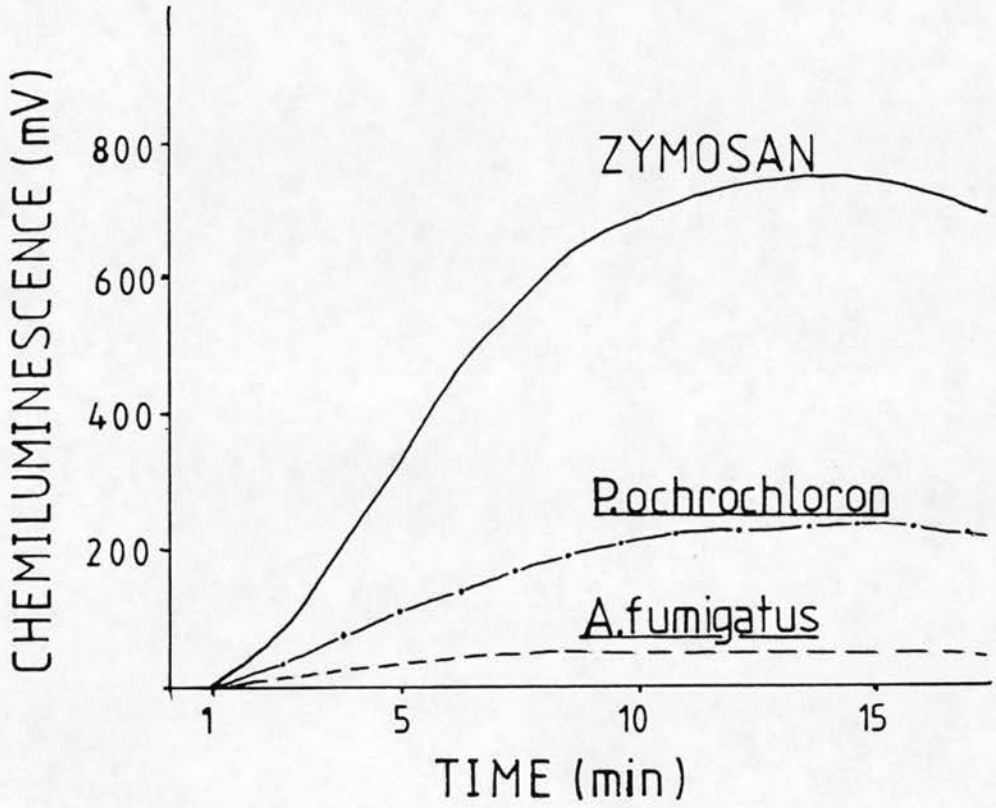


Figure 3.7 Typical profile of luminol amplified chemiluminescent responses of normal human PMN to the following triggers, opsonised in human AB sera : zymosan (5 mg), spores of A. fumigatus and spores of P. ochrochloron at a spore:cell ratio of 10:1; continuously measured in mV over a 20 min period.

TABLE 3.7 Chemiluminescent responses of monocytes and PMN to spores of A. fumigatus and P. ochrochloron expressed as peak chemiluminescence (mV) and as a percentage of the individual zymosan response.

Cell	Zymosan	A. fumigatus	P. ochrochloron	A. fumigatus	P. ochrochloron
	Peak chemiluminescence mV			% of zymosan response	
Monocyte	141.1 (16.2)	21.3 (4.6)	80.48 (21.0)	14.98 (1.35)	55.7 (10.1)
PMN	612.7 (152.3)	40.7 (11.0)	194.3 (58.1)	8.8 (2.9)	34.2 (6.1)

1 Mean (SEM) of peak chemiluminescence of cells from five subjects

2 Mean (SEM) of the results when expressed as a percentage of the zymosan response for each experiment

variance Table used for the analysis is given in the Appendix, Table A1).

3.2.3 Effect of Opsonisation of Fungal Spores

(i) Autologous versus AB serum

Opsonisation of spores in autologous serum compared with control AB serum increased the chemiluminescent responses of both monocytes and PMN (from controls) to P. ochrochloron, and PMN to A. fumigatus by approximately 24% ($p<0.05$). It had no effect on the chemiluminescence produced by monocytes in response to spores of A. fumigatus (Table 3.8)

(ii) Effect of heat inactivation

Opsonisation of spores of A. fumigatus and P. ochrochloron in autologous serum which had been heat treated for 30 min at 56°C significantly reduced the chemiluminescent responses of monocytes and PMN to both spore types. The chemiluminescence produced in response to spores which had been opsonised in sera, heat treated for 20 min at 50°C generally fell between those obtained using non-treated serum and serum heat treated at 56° for 30 min (Table 3.9).

Summary

The principal conclusion from this work is that spores of A. fumigatus elicit significantly lower chemiluminescent responses from phagocytic cells when compared with spores of P. ochrochloron or with particles of zymosan.

TABLE 3.8 A comparison of opsonisation of fungal spores in autologous versus AB serum on the chemiluminescent responses of human monocytes and PMN

Spore	Monocyte		PMN	
	AB	Autologous	AB	Autologous
A. fumigatus	14 (1.2)[1]	15 (1.2)	7 (1.4)	9 (1.5)*
P. ochrochloron	46 (1.3)	56 (1.2)*	28 (1.3)	34 (1.3)*

1 Geometric mean (SEM)[1] of the percentage of the zymosan response from 5 subjects

* Comparison between AB versus autologous $p<0.05$

TABLE 3.9 The effect of opsonisation of fungal spores in autologous serum, untreated and heat treated on the chemiluminescent responses of human monocytes and PMN

Fungal Spore	Cell	Serum Treatment None	HI50	HI56	Statistical Significance None v's HI56
A. fumigatus	Monocyte	40.2 (1.25)	20.4 (1.34)	12.8 (1.39)	$p<0.001$
"	PMN	72.6 (1.22)	64.4 (1.26)	49.2 (1.28)	$p<0.01$
P. ochrochloron	Monocyte	104.9 (1.35)	68.6 (1.27)	35.1 (1.35)	$p<0.001$
"	PMN	217.7 (1.25)	143.3 (1.22)	100.9 (1.27)	$p<0.0025$

1 Heated 50°C 20 min

2 Heated 56° 30 min

3 Geometric mean (SEM) of the mean peak chemiluminescent response (mV) from 5 subjects

3.3 The Effect of Fungal Spores on the Production of Superoxide Anion and Hydrogen Peroxide by Phagocytic Cells

The experiments using chemiluminescence suggested that spores of A. fumigatus did not elicit a significant 'respiratory burst'; the next step was to estimate the levels of the specific reactive oxygen species, superoxide anion and hydrogen peroxide, which may become release during cell-association with fungal spores.

The experiments were performed using C. parvum-stimulated mouse peritoneal exudate cells, which are known to spontaneously release high levels of reactive oxygen intermediates. In addition, the production of superoxide anion, by human monocytes and PMN challenged with fungal spores was measured.

3.3.1 Effects of fungal spores on release of superoxide anion

C. parvum-stimulated mouse peritoneal exudate cells released significantly less superoxide anion ($p<0.001$) in response to spores of A. fumigatus than in response to zymosan or spores of P. ochrochloron (Table 3.10). Both zymosan and P. ochrochloron spores were associated with a significant increase in release of superoxide anion that was significantly greater than the spontaneous release by cells ($p<0.001$). When the results from the individual spore:cell ratios were combined, P. ochrochloron was seen to stimulate an increase in superoxide anion release of about 50%, whilst zymosan, a potent trigger of superoxide anion, stimulated a 300% increase. The results are illustrated in Fig 3.8. The analyses of variance Table is given in the Appendix (Table A2).

TABLE 3.10 Release of superoxide anion by C. parvum stimulated mouse peritoneal exudate cells to spores of A. fumigatus, P. ochrochloron and zymosan opsonised in autologous serum.

Treatment	Spore:cell ratio	Superoxide anion nmol/5 x 10^5 cells/2 h
HBSS*	-	12.89 (1.20)[2]
zymosan	-	41.6 (0.60)*
A. fumigatus	10:1	12.06 (0.20)
A. fumigatus	50:1	11.07 (0.45)
A. fumigatus	100:1	11.09 (0.84)**
P. ochrochloron	10:1	14.62 (1.60)
P. ochrochloron	50:1	20.12 (1.23)
P. ochrochloron	100:1	18.4 (1.70)*

1 Treatment with Hanks' balanced salt solution (HBSS) is a measure of the spontaneous release.

2 Mean (SEM) of three to five separate experiments

* Zymosan and P. ochrochloron (all spore:cell ratios combined) elicited significantly more than HBSS $p<0.001$

** A. fumigatus (all spore ratios combined) elicited significantly less than zymosan or P.ochrochloron $p<0.001$

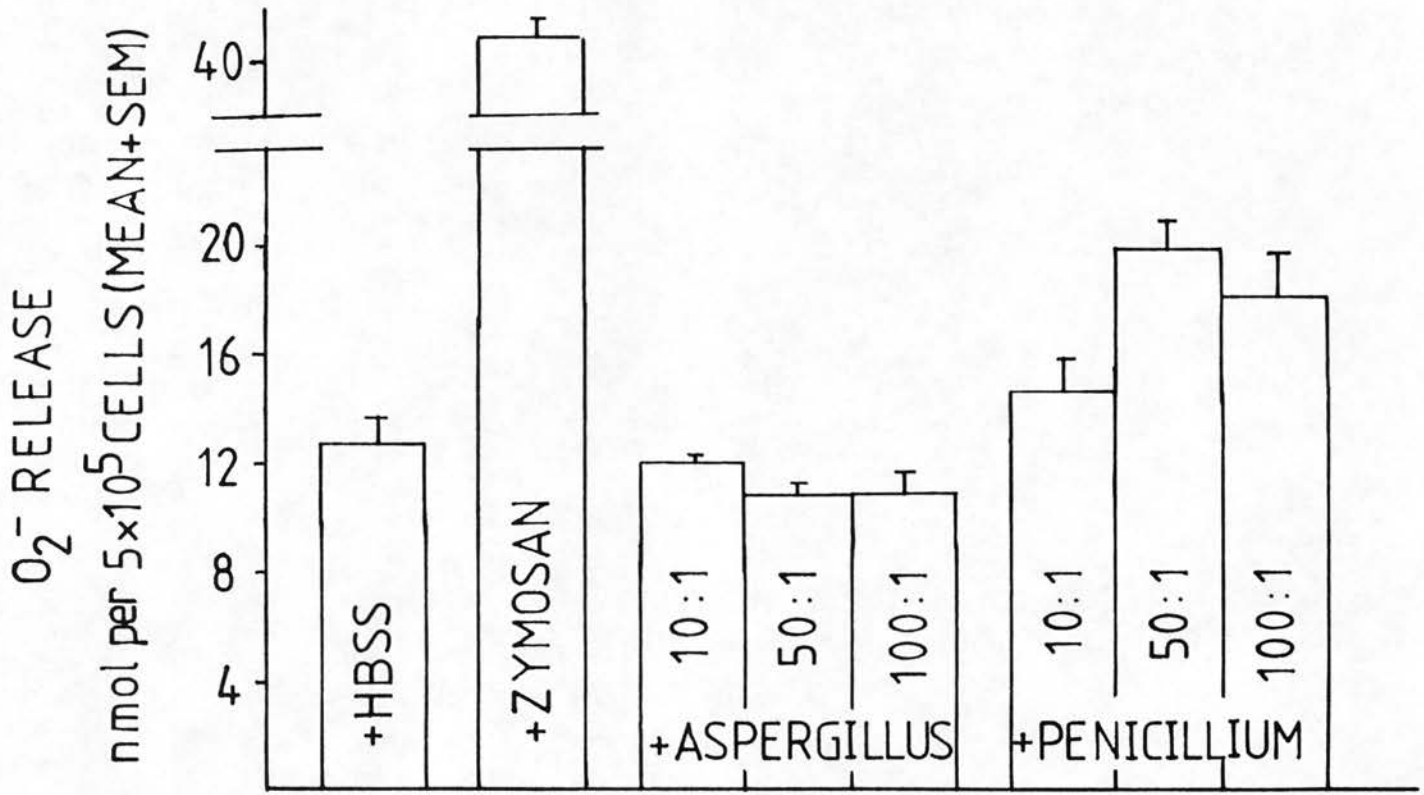


Figure 3.8 The effect of adding opsonised fungal spores of A. fumigatus and P. ochrochloron at increasing spore : cell ratio on the release of superoxide anion (O_2^-) by C. parvum-induced mouse peritoneal exudate cells (5 x 10^5 cells/2 h).

3.3.2 Effects of fungal spores on release of hydrogen peroxide

Both zymosan and P. ochrochloron slightly reduced the spontaneous release of hydrogen peroxide by C. parvum-stimulated mouse peritoneal exudate cells (Table 3.11). Spores of A. fumigatus produced a reduction of hydrogen peroxide release with increasing spore:cell ratios (Fig 3.9). When the results from the individual spore:cell ratios were combined the release of hydrogen peroxide was significantly lower in response to A. fumigatus ($p<0.001$) than to zymosan or P. ochrochloron. The analyses of variance Table is given in the Appendix (Table A3).

3.3.3 Production of superoxide anion by human monocytes and PMN in response to fungal spores

The production of superoxide anion by phagocytic cells to spores of A. fumigatus, P. ochrochloron and to zymosan, opsonised in AB serum was measured. The results in Table 3.12 show that the spontaneous release of superoxide anion by PMN is significantly higher than that produced by monocytes ($p<0.001$). Zymosan induced a significant increase in superoxide anion production in both cell types ($p<0.01$). Spores of P. ochrochloron had no significant effect on the release of superoxide anion by PMN although a slight decrease in the amount produced by monocytes was found. Spores of A. fumigatus caused a significant decrease in the spontaneous release of superoxide anion by monocytes and PMN ($p<0.002$) being considerably lower than that produced in response to spores of P. ochrochloron ($p<0.02$). The analyses of variance Table is given in the Appendix (Table A4).

TABLE 3.11 The release of hydrogen peroxide by C. parvum stimulated mouse peritoneal exudate cells to spores of A. fumigatus, P. ochrochloron and zymosan, opsonised in autologous serum.

Treatment	Spore:cell ratio	Hydrogen peroxide nmol/5 x 10^5 cells/2 h
HBSS[1]	-	10.19 (1.57)
zymosan	-	9.53 (1.53)
A. fumigatus	10:1	8.63 (1.41)
A. fumigatus	25:1	6.80 (0.35)
A. fumigatus	50:1	5.15 (0.87)*
P. ochrochloron	10:1	8.63 (1.18)
P. ochrochloron	50:1	8.16 (1.88)

1 Treatment with Hanks' balanced salt solution (HBSS) is a measure of the spontaneous release.

2 Mean (SEM) of three to five separate experiments

* A. fumigatus (all spore:cell ratios combined) elicited significantly less than P. ochrochloron and zymosan $p<0.001$

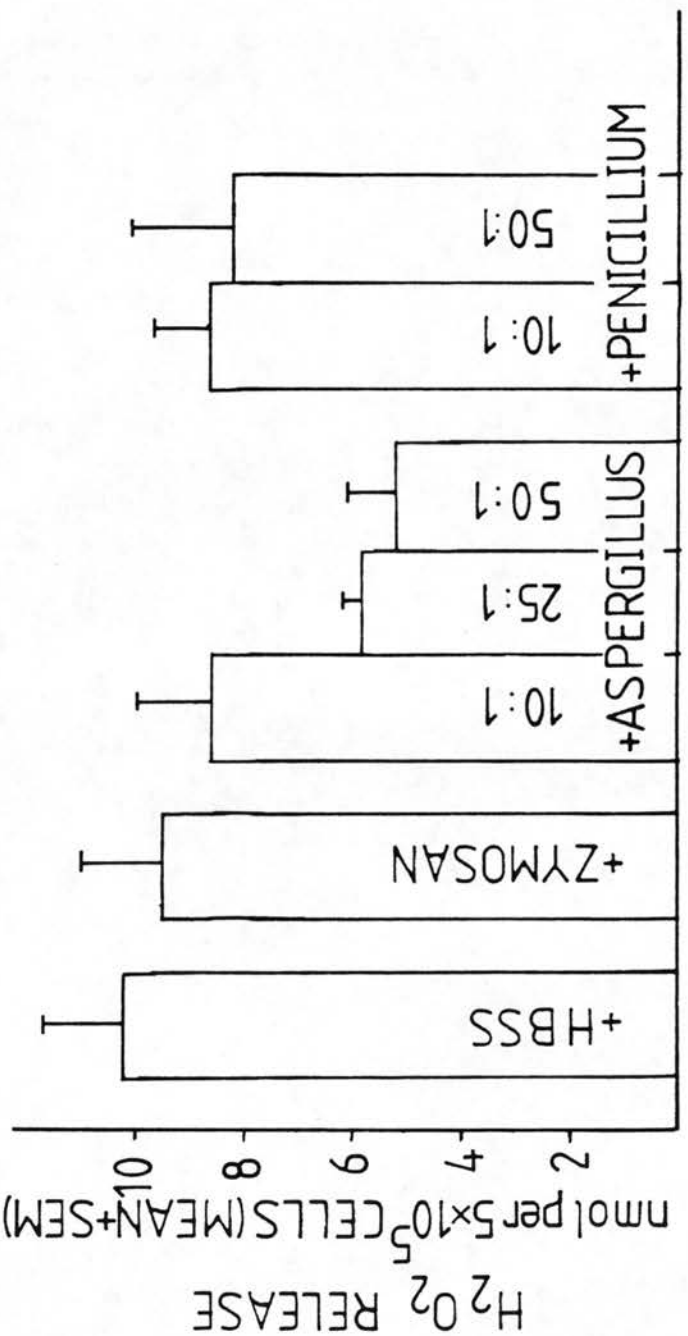


Figure 3.9 The effect of adding opsonised fungal spores of A. fumigatus and P. ochrochloron at increasing spore : cell ratio on the relgase of hydrogen peroxide (H_2O_2) by C. parvum-induced peritoneal exudate cells (5×10^5 cells/2 h).

TABLE 3.12 Comparison of the production of superoxide anion by human monocytes and PMN to spores of *A. fumigatus* and *P. ochrochloron* (spore:cell ratio 50:1) and zymosan, opsonised in AB sera.

Trigger	Monocyte	PMN
	Superoxide anion nmol 5 x 10^5 cells/2 hr	
HBSS[1]	6.46 (1.7)[2]	13.6 (4.4)*
Zymosan	13.94 (2.9)**	15.53 (4.4)**
P. ochrochloron	6.18 (2.6)	13.30 (3.9)
A. fumigatus	4.8 (1.3)***	9.9 (2.25)***

1 HBSS - Hanks' balanced salt solution measure of spontaneous release

2 Mean (SD) of results from five subjects

* PMN release significantly more than monocytes, $p<0.001$

** Responses to zymosan significantly greater than HBSS, $p<0.01$

*** Responses to *A. fumigatus* significantly less than HBSS, $p<0.002$ and to *P. ochrochloron*, $p<0.02$

Summary

The principal finding from these experiments is that spores of A. fumigatus failed to elicit production of superoxide anion and hydrogen peroxide by phagocytic cells.

3.4 Effects of Spore Diffusates on Release of Reactive Oxygen Intermediates

To examine the possibility that A. fumigatus may produce a substance that had a direct effect on cellular production of reactive oxygen intermediates, the effect of spore diffusates on the spontaneous release of reactive oxygen intermediates from C. parvum-stimulated mouse peritoneal exudate cells were measured. Diffusates from A. fumigatus greatly reduced ($p<0.001$) the spontaneous release of superoxide anion and hydrogen peroxide (Table 3.13) whilst those from P. ochrochloron did not. The analyses of variance Tables are given in the Appendix (Tables A5 and A6).

Using bronchoalveolar lavage cells obtained from rats whose lungs had been treated with C. parvum, this inhibitory effect was found to be strikingly dependent on the concentration of diffusate (Fig 3.10).

Summary

The results of these experiments have shown that spores of A. fumigatus release a substance which inhibits production of reactive oxygen intermediates by phagocytic cells.

TABLE 3.13 The effect of adding spore diffusates at a 1:4 dilution on the spontaneous release of superoxide anion and hydrogen peroxide by C. parvum stimulated mouse peritoneal exudate cells

Treatment	Superoxide anion	Hydrogen peroxide
	nmol/5 x 10^5 cells/2 h	
HBSS[1]	14.38 (0.26)[2]	18.82 (1.63)
A. fumigatus	6.73 (0.43)*	4.16 (0.52)*
P. ochrochloron	14.40 (0.67)	19.43 (2.25)

1 The addition of Hanks' balanced salt solution (HBSS) is a measure of the spontaneous release.

2 Mean (SEM) of three separate experiments. Response to A. fumigatus significantly lower than HBSS or P. ochrochloron $p<0.001$

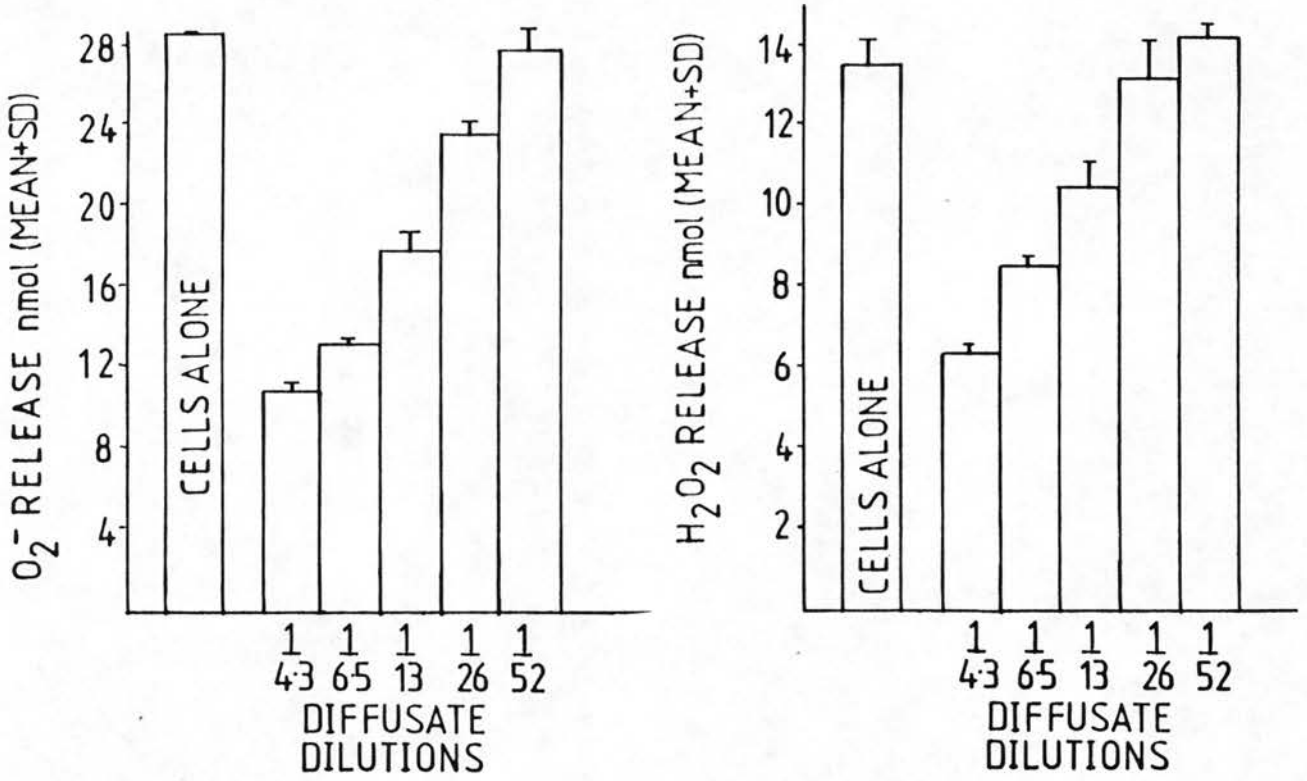


Figure 3.10 The effect of increasing dilution of spore diffusates of A. fumigatus diluted in Hanks' balanced salt solution (HBSS) on the spontaneous release of superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) by phorbol myristate acetate-triggered C. parvum-induced rat bronchoalveolar lavage cells (2.5 x 10^5 cells/2 h) when compared with cells alone (HBSS added).

3.4.1 Direct effect of spore diffusates on superoxide anion and hydrogen peroxide

In order to examine whether the diffusate was influencing production of reactive oxygen intermediates or was scavenging the reactive oxygen intermediates once produced, the diffusate at a 1:4 dilution was added to a cell free superoxide anion generation system and to hydrogen peroxide solutions.

(i) Superoxide anion

Superoxide anion was generated by the xanthine oxidase-acetaldehyde system. The addition of spore diffusates of A. fumigatus to superoxide anion did not affect the measurable amount of superoxide anion; the amount present remained the same as that found with HBSS (Table 3.14). However, the addition of superoxide dismutase (which is used in the conversion of superoxide anion to hydrogen peroxide) removed the superoxide anion from the system. This result confirmed that the diffusate was not scavenging superoxide anion.

(ii) Hydrogen peroxide

Similarly, the diffusates did not scavenge hydrogen peroxide. The addition of control HBSS to a system containing hydrogen peroxide resulted in a linear increase in absorbance at 610 nm (a measure of hydrogen peroxide in the system) with increasing concentration of hydrogen peroxide (1-20 nmol) shown in Fig 3.11. The addition of the diffusate to hydrogen peroxide resulted in an increase in absorbance at 610 nm, the same as that given with HBSS. However, when catalase, an enzyme which breaks down hydrogen peroxide, was added to the system no increase in absorbance at 610 nm was obtained.

TABLE 3.14 The effect of adding diffusates of *A. fumigatus* at a 1/4 dilution on superoxide anion generated by the xanthine oxidase-acetaldehyde generation system when compared with the addition of control HBSS or HBSS and superoxide dismutase

Treatment	Superoxide anion nmol
HBSS	5.86 (0.63)[1]
A. fumigatus diffusate	5.84 (0.38)
HBSS + superoxide dismutase	0

1 Mean (SD) of results of two experiments

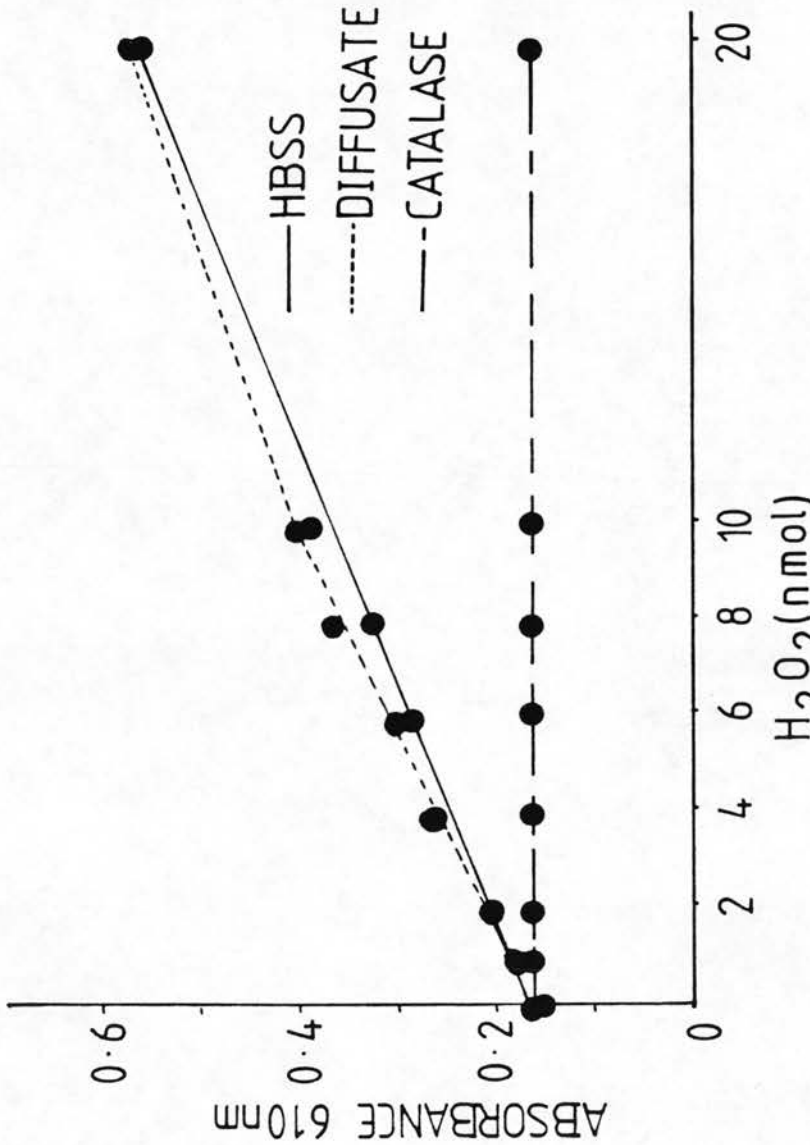


Figure 3.11 The absorbance of the horseradish peroxidase - phenol red solution at 610 nm following incubation for 30 min at 37°C with hydrogen peroxide (1-10 nmol) which had been previously incubated (30 min 37°C) with either HBSS (control) catalase (100 μg) or *A. fumigatus* diffusate 1/4 dilution.

The results of these experiments have shown that spore diffusates of A. fumigatus do not scavenge the reactive oxygen intermediates superoxide anion and hydrogen peroxide.

3.5 Time Course of Production of Spore Diffusates of A. fumigatus

In order to study the kinetics of diffusate release to the supernatant, the effect of time on the inhibition of reactive oxygen intermediate production was examined. Table 3.15 demonstrates that washings (2 min incubation) from the spores significantly reduced reactive oxygen intermediate release ($p<0.001$) by C. parvum-stimulated mouse peritoneal exudate cells. Therefore, the diffusate is released immediately the spores are put into liquid. In addition the spores, once washed, continued to release the diffusate. The longer the spores were incubated, the greater was the inhibitory capacity of the diffusate.

Summary

The component of the spore diffusate which inhibits the production of reactive oxygen intermediates by phagocytic cells is released immediately the spores are put into solution.

3.6 Effect of Spore Diffusates of A. fumigatus on Cell Viability

3.6.1 Trypan blue exclusion

Viability studies using trypan blue exclusion established that the spore diffusates were not having a toxic effect on the cells as the percentage viability of the phagocytic cells (human monocytes, PMN, alveolar macrophages and mouse macrophages) after an incubation

TABLE 3.15 The effect of time on the diffusion, from A. fumigatus spores, of activity which inhibits the production of superoxide anion and hydrogen peroxide by C. parvum stimulated mouse peritoneal exudate cells

Treatment	Superoxide anion	Hydrogen peroxide
	nmol/5 x 10^5 cells/2 h	
HBSS[1]	13.43 (0.42)[2]	13.9 (0.69)
2 min incubation (washings)	6.93 (0.25)*	8.6 (0.36)*
washed spores + 3 hr incubation	5.13 (0.18)*	4.43 (0.14)*
3 hr incubation	4.77 (0.15)*	3.3 (0.06)*

1 Hanks' balanced salt solution (HBSS) is a measure of the spontaneous release.

2 Mean (SEM) of results from three experiments

* All treatment significantly inhibited the spontaneous release of superoxide anion and hydrogen peroxide $p < 0.001$

period in spore diffusates for 2 h was the same as that found with control HBSS (>95% viable).

3.6.2 ^{51}Chromium release assay

The estimation of ^{51}Cr release is a sensitive technique for measuring the potential toxicity of substances towards cells. The results in Table 3.16 show that spore diffusates of A. fumigatus did not increase the spontaneous release of ^{51}Cr by human pulmonary macrophages following incubation for three hours in vitro.

Summary

These results confirm that spore diffusates are not directly toxic to phagocytic cells.

3.7 Preliminary Characterisation of Diffusates from A. fumigatus spores

3.7.1 Molecular weight

The inhibitory effect of the diffusate on reactive oxygen intermediate release by C. parvum-stimulated mouse peritoneal exudate cells was removed by dialysis (Table 3.17), indicating that the molecular weight of the active substance(s) in the diffusate is less than 14,000 daltons.

Further studies which involved passing the diffusate through special filters with molecular weight exclusion limits of 1,000, 5,000 and 10,000 daltons were performed. The initial sample and the filtrates were then tested for their capacity to inhibit the production of superoxide anion by C. parvum stimulated mouse peritoneal exudate

TABLE 3.16 The effect of spore diffusates of A. fumigatus on the release of ^{51}Chromium by radio-labelled human pulmonary macrophages

Treatment	Alveolar macrophage ^{51}Cr release cpm
HBSS[1]	2846 (998)[2]
Diffusate (1/2 dilution)	2587 (451)
Diffusate (1/4 dilution)	2394 (1031)
Distilled water 0.01% Triton X[3]	24302 (4671)

1 Hanks' balanced salt solution - a measure of the spontaneous release

2 Mean (SD) of 5 replicates

3 Measure of the total ^{51}Cr taken up by the macrophages

TABLE 3.17 The effect of dialysis on the capacity of A. fumigatus diffusate to inhibit the production of superoxide anion and hydrogen peroxide by C. parvum stimulated mouse peritoneal exudate cells

Treatment	Superoxide anion	Hydrogen peroxide
	nmol/5 x 10^5 cells/2 h	
HBSS[1]	12.98 (1.38)[2]	21.3 (2.06)
diffusates	5.65 (0.57)	8.75 (1.07)
diffusates incubated $4^{o}C$ 18 h	9.95 (1.28)	13.86 (0.38)
diffusates dialysed $4^{o}C$ 18 h	13.76 (1.69)*	25.6 (1.82)*

1 Hanks' balanced salt solution is a measure of the spontaneous release.

2 Mean (SEM) of three experiments

* Statistical significance between means of diffusates incubated $4^{o}C$ 18 h versus dialysed diffusate is $p<0.0001$

cells. This experiment showed that the inhibitory component has a molecular weight between 1,000 and 5,000 daltons (Table 3.18).

3.7.2 Biochemical characterisation

The biochemical characterisation of the spore diffusate, is being carried out by Dr V Hearn of the Public Health Laboratories, Colindale, London. Her preliminary results show that the diffusate contains an aromatic compound which is eluted on high performance liquid chromatography at 254 nm. Amino acids have been detected and the predominant ones are arginine, serine, glutamine, aspartic acid, glycine, alanine and valine. Further characterisation of the spore diffusate is in progress.

Summary

The inhibitory component of spore diffusates of A. fumigatus has a molecular weight of between 1,000 and 5,000 daltons. The exact biochemical nature of the substance is still to be established.

3.8 The Effect of Spore Diffusates on the Phagocytosis of Sheep Red Blood Cells

The previous results have shown (section 3.1) that spores of A. fumigatus appear to be resistant to ingestion. In an attempt to assess accurately the effect of spore diffusates of A. fumigatus and P. ochrochloron on phagocytosis of antibody coated ^{51}Cr labelled sheep red blood cells by C. parvum-stimulated mouse peritoneal exudate cells was measured. This technique has the advantage that all extracellular (non-ingested) sheep red blood cells, including those attached to the cell surface, are removed by lysis with red

TABLE 3.18 Effect of molecular sieving on the capacity of A. fumigatus diffusate to inhibit superoxide anion production by C.parvum-stimulated mouse peritoneal exudate cells

Treatment	Superoxide anion nmol/5 x 10^5 cells/2 h
HBSS[1]	7.33 (0.44)[2]
Spore diffusate (before filtration)	1.27 (0.43)
Filtrate < 1,000	7.90 (0.58)
Filtrate < 5,000	1.43 (0.35)
Filtrate <10,000	2.41 (0.06)

1 Hanks' balanced salt solution a measure of the spontaneous release

2 Mean (SD) of 3 replicates

blood cell lytic buffer, thereby enabling an accurate measurement of phagocytosis to be made. There was significantly more phagocytosis ($p<0.0005$) of the sheep red blood cells coated in specific anti-sheep red blood cell than of those coated with irrelevant mouse IgG (Table 3.19). This result established the validity of the technique. The ^{51}Cr labelled sheep red blood cells coated in specific anti-sheep red blood cell antibody were used to assess the effects of the spore diffusates. Diffusates of A. fumigatus inhibited phagocytosis by about 63% ($p<0.0025$) whilst spore diffusates of P. ochrochloron had no significant effect (Table 3.19). As a positive control cytochalasin B (a known inhibitor of phagocytosis) was included; this inhibited the phagocytosis of radiolabelled red blood cells coated with specific antibody to sheep red blood cells by 44% ($p<0.005$) at a concentration of 1.25 µg.

Summary

Spore diffusates inhibit the phagocytosis of radiolabelled antibody coated sheep red blood cells.

3.9 Effect of Spore Diffusates on Phagocytic Cell Movement

The effect of spore diffusates on the chemotaxis and cytoplasmic spreading of phagocytic cells was measured.

3.9.1 Effect of spore diffusates on chemotaxis of PMN

Spore diffusates of A. fumigatus inhibited the number of cells migrating towards the chemoattractant (zymosan activated serum) by approximately 46% $p<0.001$). Spore diffusates of P. ochrochloron had no

TABLE 3.19 The effect of spore diffusates at a 1:2 dilution on the phagocytosis of antibody-coated radiolabelled-sheep red blood cells by C. parvum stimulated mouse peritoneal exudate cells incubated for 1.5 h at 37°C.

Treatment	Antibody coating of ^{51}Cr srbc	Phagocytosis (cpm) Mean (SEM)
HBSS[1]	Mouse IgG	87.1 (10.04)[2]
HBSS	αsrbc	440.5 (36.6)
A. fumigatus diffusate	αsrbc	161.8 (29.4)**
P. ochrochloron diffusate	αsrbc	408.3 (51.6)
cytochalasin B (1.25 ug)	αsrbc	247.8 (34.2)*

1 Hanks' balanced salt solution

2 Means (+ SEM) of three separate experiments

Significantly less than HBSS αsrbc
* $p<0.005$ ** $p<0.0025$

inhibitory effect on the migration of the cells (Table 3.20) illustrated in Figs 3.12 a-c. Further experiments which involved measuring the random migration of the cells in HBSS alone in the top compartment versus A. fumigatus diffusates in the lower compartment (Table 3.21) confirmed that the spore diffusates of A. fumigatus were not acting as a chemoattractant. A comparison of the effect of 8 different preparations of A. fumigatus diffusate from isolates of the same strain on the chemotaxis of human PMN towards the known chemoattractant, zymosan activated serum, showed that this inhibitory activity was reproducible ($p<0.001$) (see Table 3.22). The analysis of variance Table is given in the Appendix (Table A7).

3.9.2 Effect of spore diffusates on phagocytic cell spreading

Spore diffusates of A. fumigatus were seen to inhibit the spreading of C. parvum-stimulated mouse peritoneal exudate cells when compared with control HBSS and with spore diffusates of P. ochrochloron (Fig 3.13 a-c). Measurement of the mean diameters of the cells showed that those cells treated with A. fumigatus diffusate were substantially less spread (by approximately 54%) than those with control HBSS (Table 3.23). The differences between cells treated with A. fumigatus diffusate and the controls were significant (both $p<0.001$). The analysis of variance Table is given in the Appendix (Table A8).

Summary

The results so far have shown that spores of A. fumigatus appear to resist ingestion, inhibit the 'respiratory burst' and produce a substance which also inhibits phagocytosis, production of reactive

TABLE 3.20 The effect of spore diffusates of A. fumigatus and P. ochrochloron on the migration of human PMN towards zymosan activated serum through 3 μm filters following incubation at 37°C for 45 min

Experiment	HBSS[1] control	A. fumigatus diffusate	P. ochrochloron diffusate
1	68.5 (4.8)[2]	32.6 (3.4)*	68.4 (6.0)
2	62.9 (1.2)	36.2 (4.8)*	62.6 (0.0)
3	83.3 (7.6)	46.2 (4.7)*	ND[3]

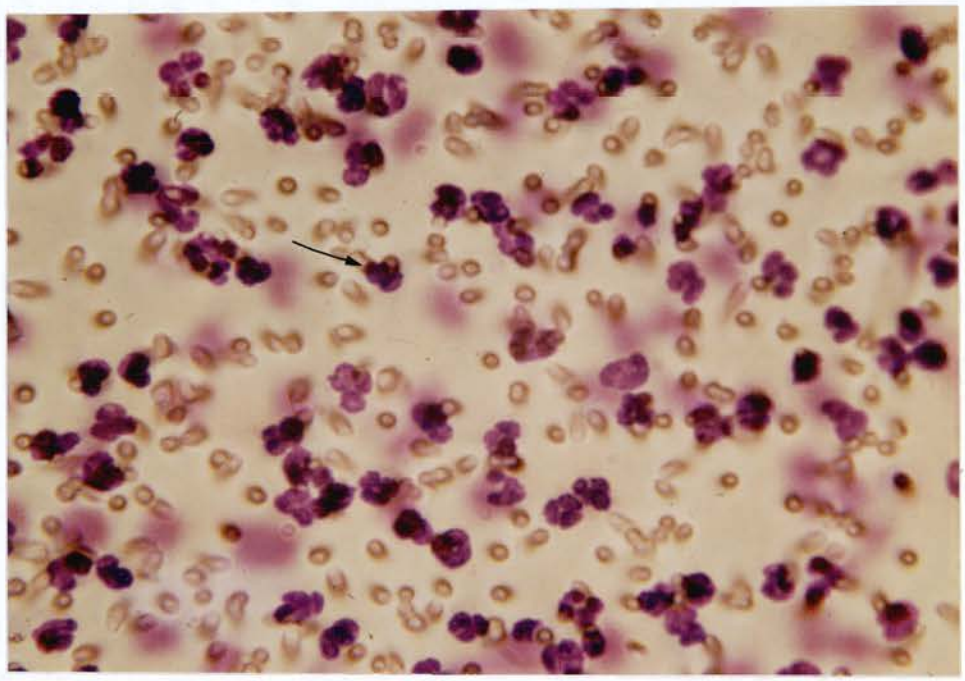
1 Hanks' balanced salt solution

2 Results of three separate experiments expressed as the mean (SD) of the number of cells migrated per 5 high power fields.

3 ND = not done

* Significantly lower than HBSS $p<0.001$

(a)



(b)

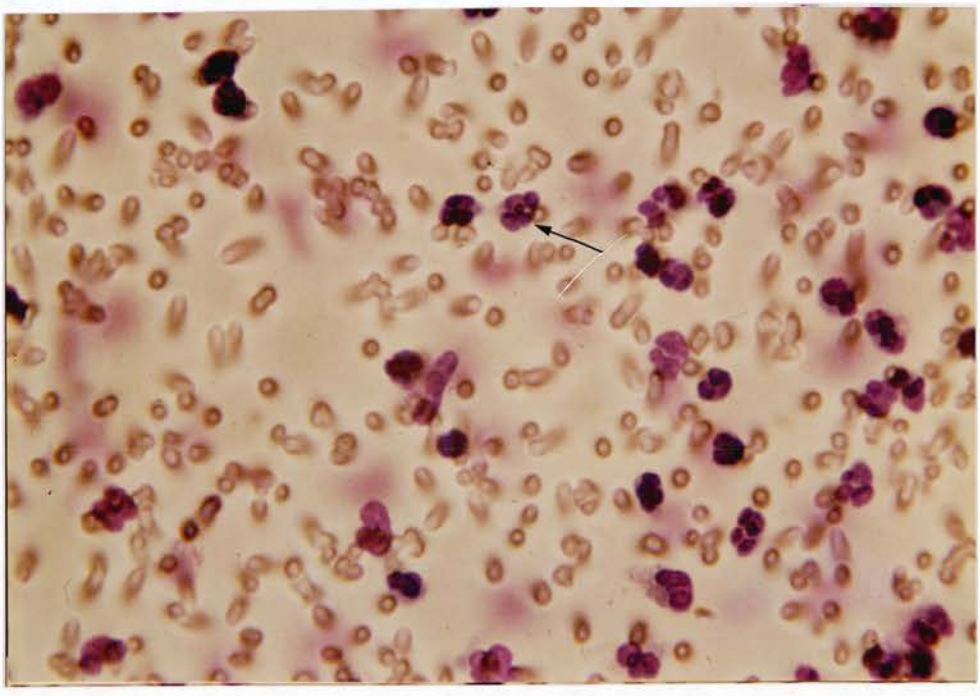


Figure 3.12 a-b Photomicrographs of the number of human PMN which have migrated towards zymosan activated serum following incubation at 37° for 45 min. The effect of adding (a) HBSS, (b) A. fumigatus diffusate (1/2 dilution). The cells which have migrated are the darkly staining ones highlighted by arrows. Original magnification x 400. The small brown fringed circles are the 3μm pores of the filter membrane.

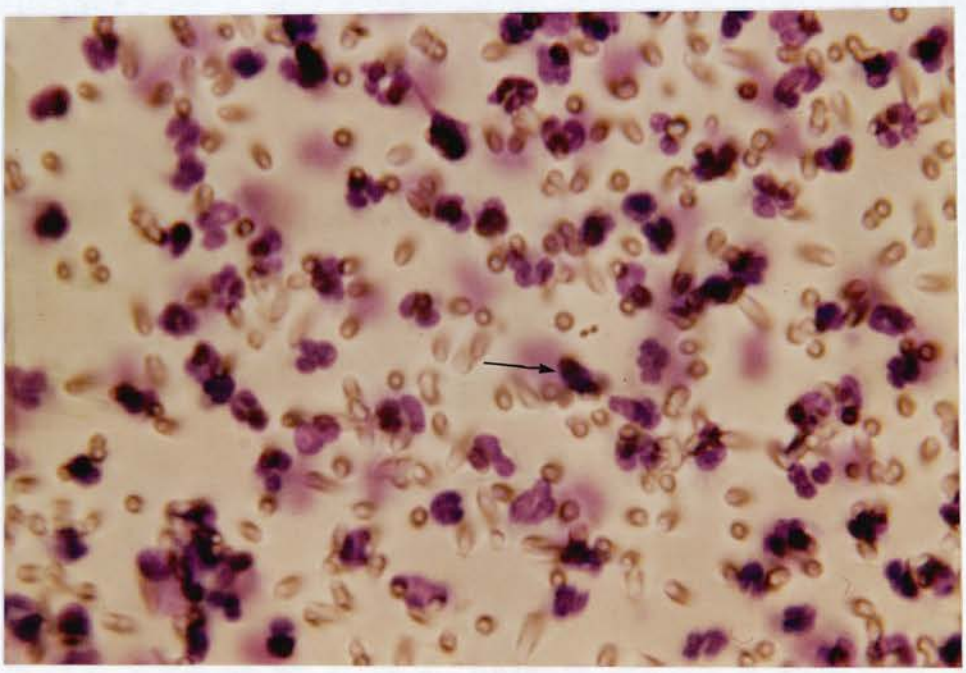


Figure 3.12 c A photomicrograph of the number of human PMN which have migrated towards zymosan activated serum following incubation at 37°C for 45 min. The effect of adding (c) P. ochrochloron diffusate (1/2 dilution). The cells which have migrated are the darkly staining ones highlighted by arrows. Original magnification x 400.

TABLE 3.21 Effect of A. fumigatus diffusates on random migration of human PMN cells in a blindwell chamber incubated for 45 min at 37°C

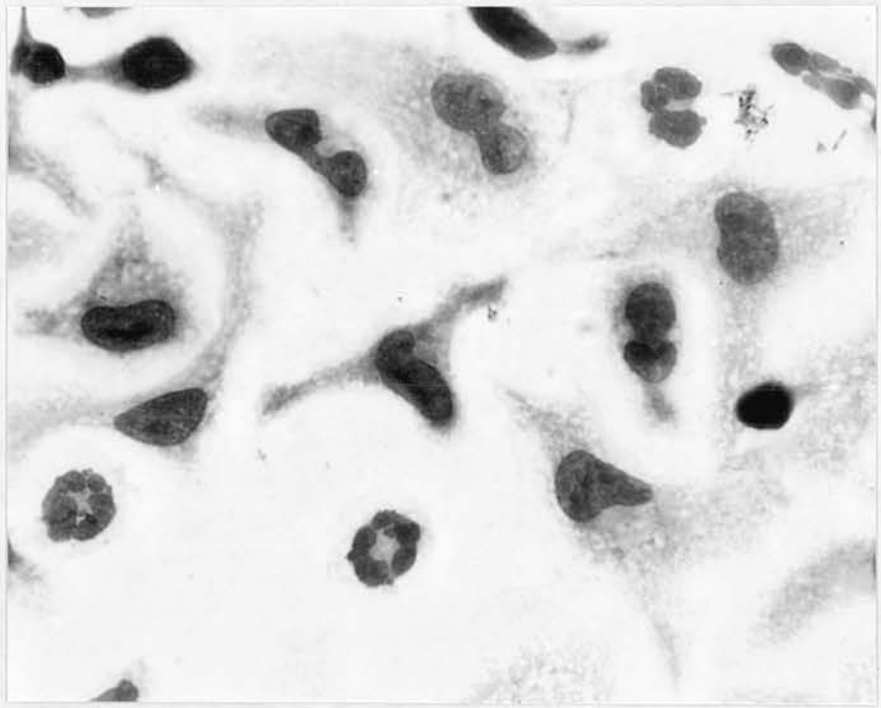
Treatment		No. of cells migrated per 5 high power fields
Top compartment	Lower compartment	
HBSS[1]	HBSS	3.0 (1.01)[2]
A. fumigatus diffusate	A. fumigatus diffusate	1.53 (0.39)
HBSS	A. fumigatus	2.73 (0.43)

1 Hanks' balanced salt solution

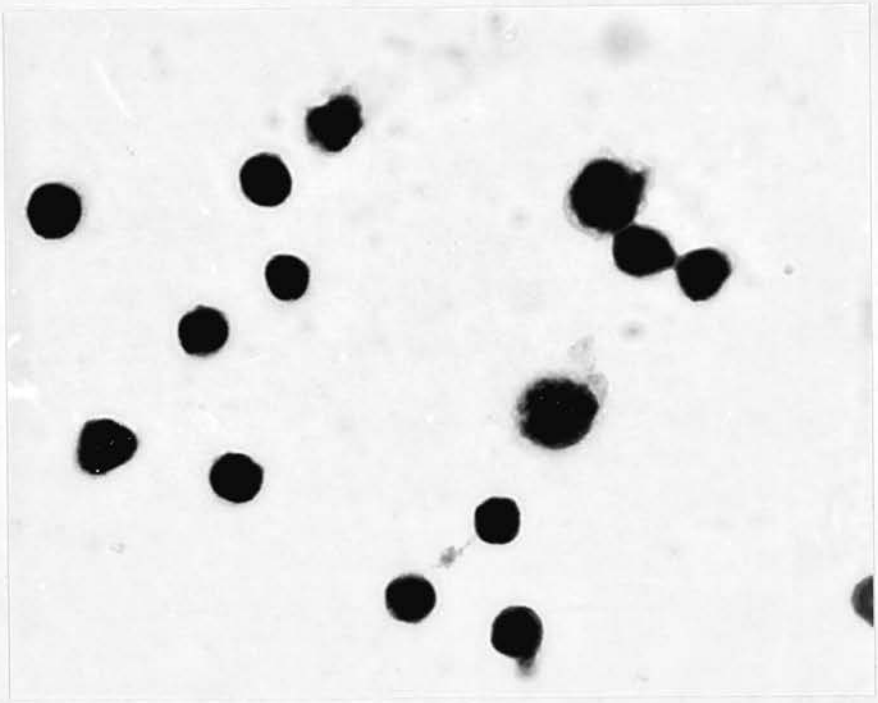
2 Mean (SEM) of two experiments

TABLE 3.22 The effect of 8 different preparations of A. fumigatus diffusate on the migration of human PMN towards zymosan activated serum through a 3 µm filter in a blindwell chamber following incubation for 45 min at 37°C

A. fumigatus diffusate preparation	% of inhibition of migration
1	52.7
2	44.0
3	41.8
4	50.3
5	56.6
6	44.5
7	49.0
8	50.9



(a)



(b)

Figure 3.13 a-b Photomicrographs of the spreading of C. parvum stimulated mouse peritoneal exudate cells following incubation at 37°C for 1 h in RPMI-10% foetal calf serum containing (a) HBSS, (b) A. fumigatus diffusate (1/2 dilution) Original magnification x 600.

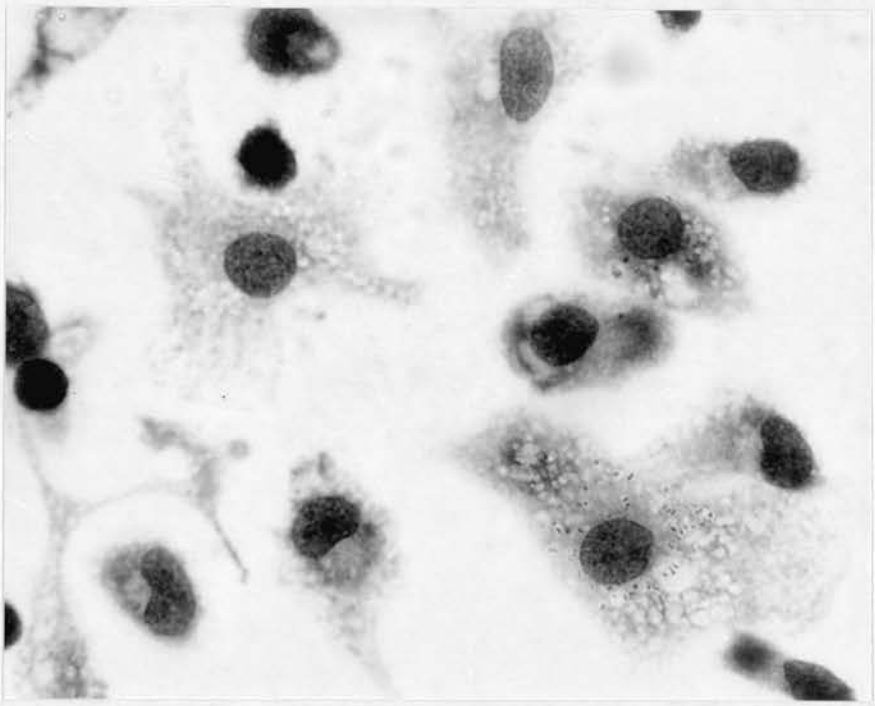


Figure 3.13 c A photomicrograph of the spreading of C. parvum stimulated mouse peritoneal exudate cells following incubation at 37°C for 1 h in RPMI-10% foetal calf serum containing (c) P. ochrochloron diffusate (1/2 dilution) Original magnification x 600.

TABLE 3.23 The effect of spore diffusates on the spreading of *C. parvum*-stimulated mouse peritoneal exudate cells

Treatment	Cell diameter Mean (SD)
HBSS control[1]	20.2 (2.9)[2]
A. fumigatus diffusate	9.2 (0.5)*
P. ochrochloron diffusate	19.3 (2.6)

1 Hanks' balanced salt solution

2 Mean (SD) of three separate experiments

* Significantly less than HBSS $p<0.001$

oxygen intermediates and cell movement.

3.10 Killing of Fungal Spores by Rat Alveolar Macrophages

In order to put of the above findings into perspective the next stage was to examine the overall ability of phagocytic cells to deal with spores of A. fumigatus by measuring their capacity to kill the fungus.

The killing assay involved incubating spores of phagocytic cells for 3 h at 37°C. The spores were distrupted from the cells and the colony forming units (CFU) measured. The results given in Table 3.24 show that spores of A. fumigatus opsonised in autologous sera were significantly more resistant to killing in vitro by rat alveolar macrophages, than were similarly opsonised spores of P. ochrochloron ($p<0.01$) even although the number of spores becoming cell-associated was similar; percentage mean (+ SEM): A. fumigatus - 86.3 ($\pm$ 3.5) P. ochrochloron - 83.6 ($\pm$ 2.9).

Summary

Spores of A. fumigatus opsonised in autologous sera are more resistant to killing by human pulmonary macrophages than similarly opsonised spores of P. ochrochloron.

3.11 Interaction of Fungal Spores with Human Lung Macrophages

The previous results have shown that macrophages from mice and rats are less efficient at killing spores of A. fumigatus than spores of P. ochrochloron. The next stage was to see if these differences

TABLE 3.24 Killing of fungal spores, opsonised in autologous serum, by rat alveolar macrophages incubated at 37°C for 3 h

Experiment	A. fumigatus	P. ochrochloron
	% killed	
1	11.5 (0.60)[1]*	16.5 (1.5)
2	25.1 (1.52)*	33.5 (5.5)
3	15.9 (0.78)*	34.2 (0.41)

1 Mean (SD)

* % of A. fumigatus killed significantly less than P. ochrochloron $p<0.01$

could be found using human pulmonary macrophages.

3.11.1 Cell-association

More than 60% of the opsonised fungal spores became cell-associated with human pulmonary macrophages following incubation for one hour at 37°C (Table 3.25). There were no significant differences in the percentage of spores of A. fumigatus becoming cell-associated when compared with spores of P. ochrochloron.

3.11.2 Killing

Spores of A. fumigatus opsonised in 5% pooled normal sera, were significantly more resistant to killing by human pulmonary macrophages than similarly opsonised spores of P. ochrochloron ($p<0.02$, Fig 3.14). Approximately 46% more spores of P. ochrochloron were killed by the macrophages than were spores of A. fumigatus.

3.11.3 Effect of serum treatment on spore killing

The ability of pulmonary macrophages to kill spores which had been opsonised in heated sera was examined. Sera which had been heated to 50°C for 20 min prior to opsonisation had no significant effect on macrophage killing of fungal spores. However, sera which had been heated to 56°C for 30 min prior to opsonisation significantly increased by (approximately 74%) the ability of pulmonary macrophages to kill spores of A. fumigatus when compared with non-heat-treated sera ($p<0.001$, Table 3.26). The percentage killing of P. ochrochloron remained the same. The analyses of variance Table is given in the Appendix (Table A9).

TABLE 3.25 The percentage of spores opsonised in pooled normal serum which were cell-associated with human pulmonary macrophages after incubation at 37°C *in vitro*

Fungal spore	% of cell-associated spores
A. fumigatus	62.7 (3.7)[1]
P. ochrochloron	65.3 (4.5)

1 Mean (SEM) of results from 10 subjects

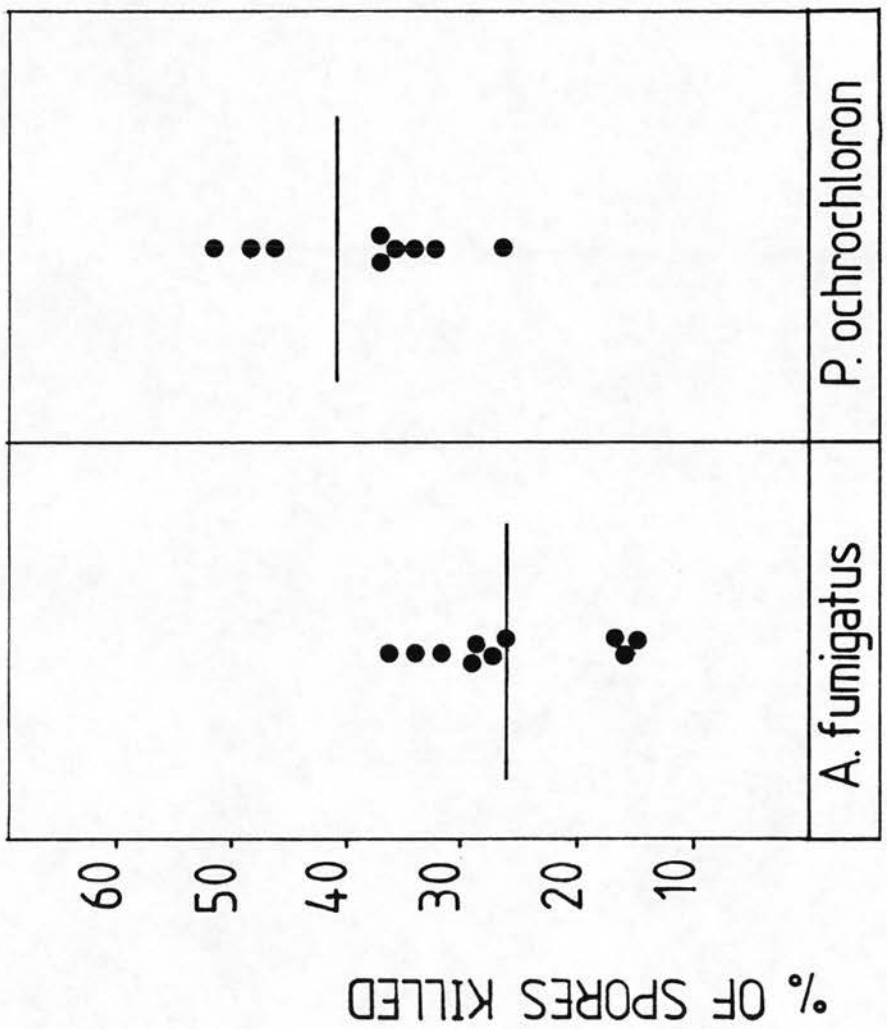


Figure 3.14 The percentage of spores of A. fumigatus and P. ochrochloron, opsonised in 5% pooled normal serum, which were killed by human pulmonary macrophages (n = 10) following incubation at 37°C for 3 h at a spore:cell ratio of 1:1. Significantly more spores of P. ochrochloron were killed when compared with A. fumigatus $p<0.02$

TABLE 3.26 The effect of heating the opsonising sera on the killing of fungal spores by human pulmonary macrophages after incubation at 37°C for 5 h

Spore	Serum Treatment	% of spores killed
A. fumigatus	None	27.09 (3.23)[1]*
A. fumigatus	50°C, 20 min	30.01 (4.16)
A. fumigatus	56°C, 30 min	47.02 (4.35)**
P. ochrochloron	None	39.7 (3.29)
P. ochrochloron	50°C, 20 min	42.56 (3.32)
P. ochrochloron	56°C, 30 min	39.83 (7.64)

1 Mean (SEM) of results from 10 subjects

* A. fumigatus significantly more resistant to killing than P. ochrochloron $p<0.02$

** Significantly more spores of A. fumigatus killed when opsonised in 5% heat-treated serum (56°C 30 min) compared with 5% untreated sera $p<0.001$

3.11.4 Effect of increasing concentration of serum on spore killing

Spores of both A. fumigatus and P. ochrochloron were opsonised in concentrations of pooled normal sera ranging from 1-25%. Serum was used both untreated and after heating for 30 min at 56°C.

(i) Opsonisation in untreated serum

The results (Fig 3.15) show that increasing concentrations of untreated sera significantly reduced ($p<0.001$) the ability of human pulmonary macrophages to kill spores of A. fumigatus. Human pulmonary macrophages were unable to kill spores of A. fumigatus opsonised in 10% sera and even appeared to enhance the germination of spores opsonised in 25% sera when compared with control spores alone. Decreased killing with increasing concentrations of opsonising sera was also found with spores of P. ochrochloron, the largest decrease falling between the 1% and 5% serum concentrations ($p<0.025$). However, unlike A. fumigatus spores of P. ochrochloron were still being killed, by human pulmonary macrophages at a serum concentration of 25%, (20% killing occurred).

(ii) Opsonisation in heat treated serum

In contrast to the results found using untreated serum, opsonisation of fungal spores in increasing concentrations of serum, which had been heat-treated, had no effect on the killing of A. fumigatus by human pulmonary macrophages (Fig 3.16). Similarly, no real differences in the killing of P. ochrochloron opsonised in heat-treated serum were found except a decrease in killing between the 1% and 5% concentration, an effect which paralleled that found with P. ochrochloron using untreated sera.

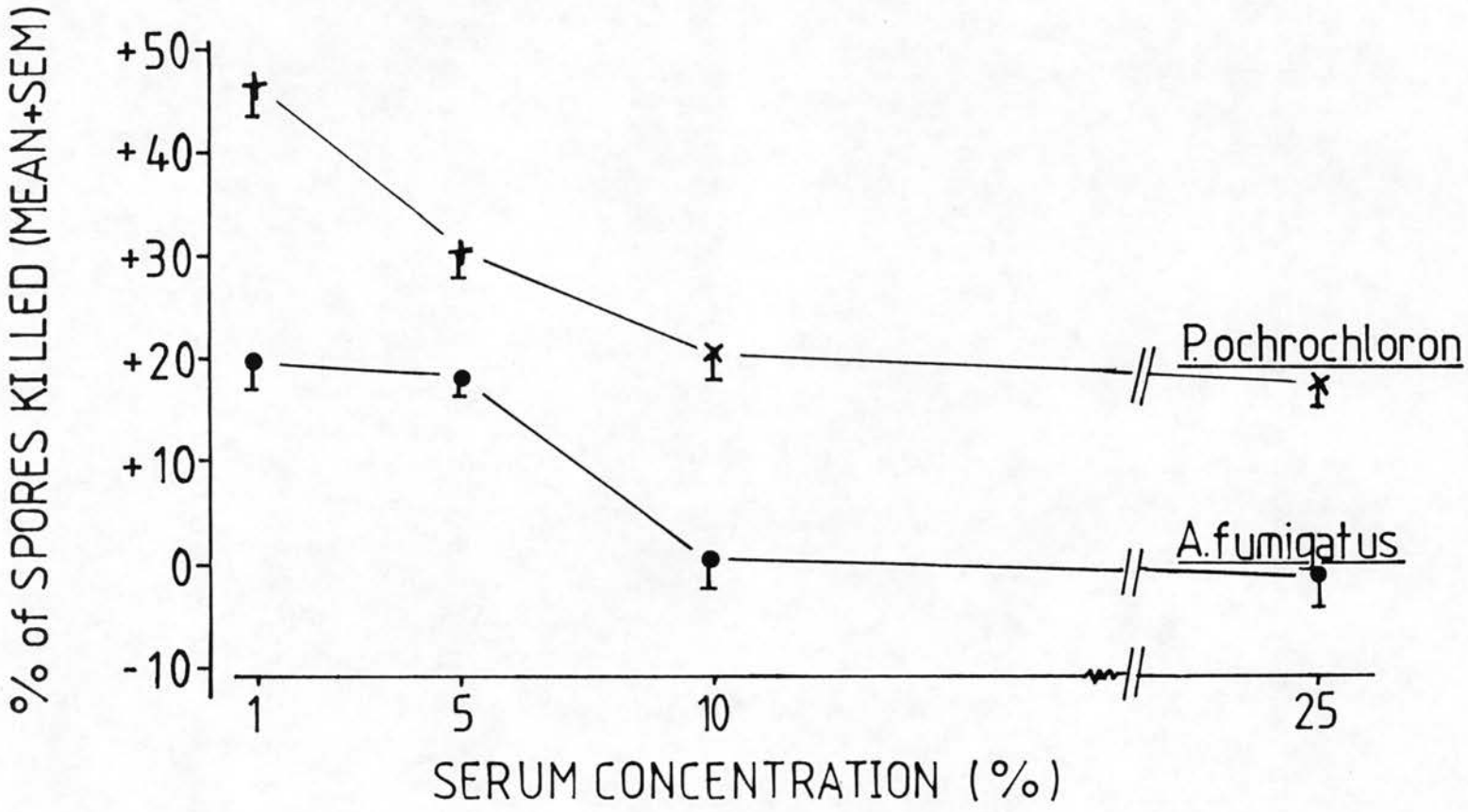


Figure 3.15 The effect of increasing concentrations of pooled normal serum (1-25%) on the killing of spores of A. fumigatus and P. ochrochloron by human pulmonary macrophages following incubation at 37°C for 3 h at a spore:cell ratio of 1:1. Results shown are the mean (SEM) of two experiments.

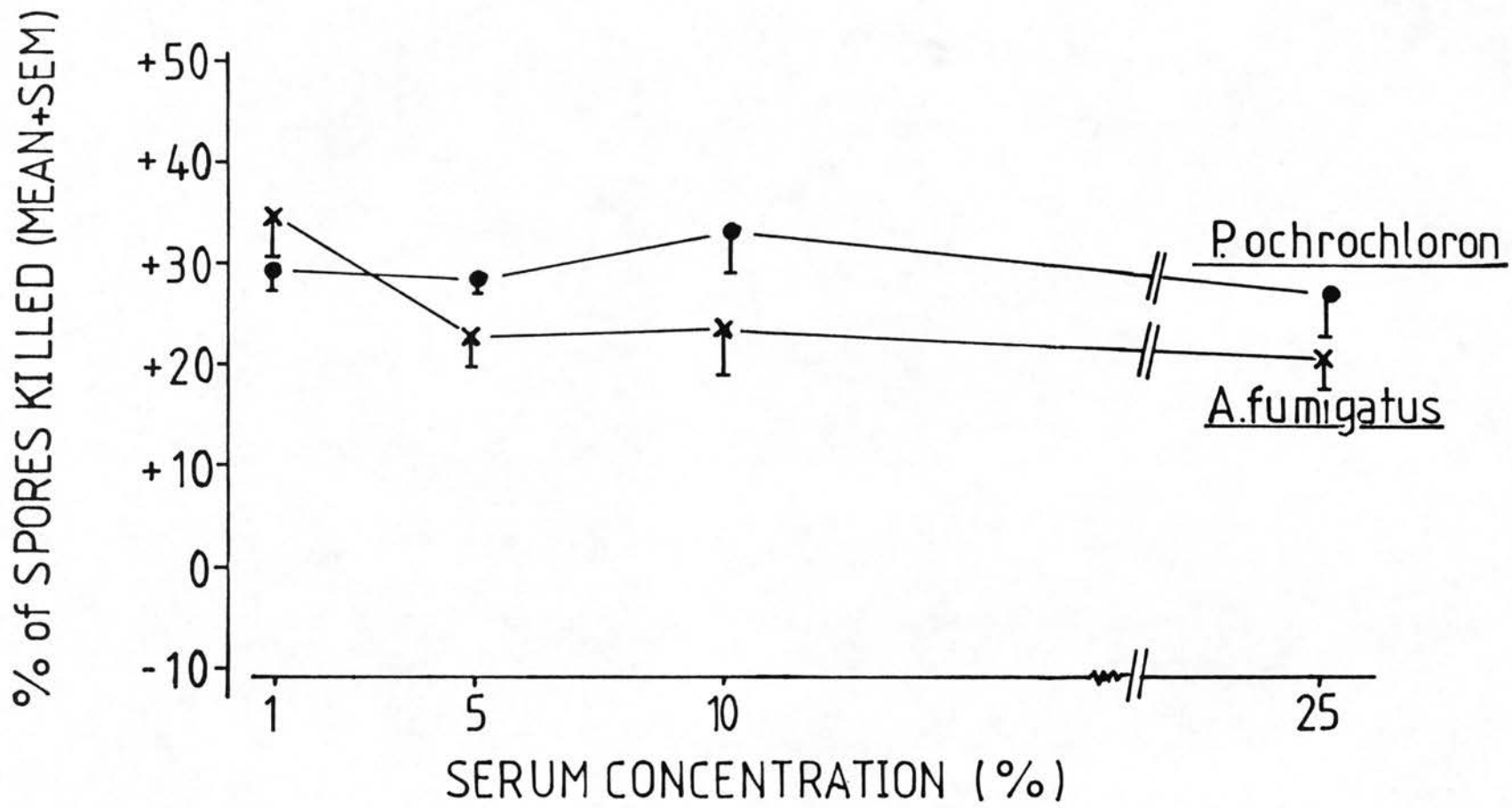


Figure 3.16 The effect of increasing concentrations of pooled normal serum, which had been heat-treated (30 min 56°C) on the killing of spores of A. fumigatus and P. ochrochloron by human pulmonary macrophages following incubation at 37°C for 3 h at a spore:cell ratio of 1:1. Results shown are the mean (SEM) of two experiments.

Therefore, serum contains a heat labile component which enables spores of A. fumigatus to resist killing by human lung macrophages. No such effect was found with spores of P. ochrochloron.

3.12 Effect of Opsonisation in Sera Containing Specific Antibody on the Killing of A. fumigatus by Mouse Macrophages

Sera and peritoneal macrophages obtained from naive mice and mice specifically sensitised to A. fumigatus were used for this experiment. Although sera containing specific antibody to A. fumigatus appeared to enhance the ability of mouse peritoneal macrophages from naive animals to kill spores of A. fumigatus the effect was not statistically significant (Table 3.27). The cells from mice specifically sensitised to A. fumigatus killed slightly more spores, opsonised in naive sera (36.4%), when compared with the percentage killing found with naive cells (32.7%). Opsonisation in specific antibody did not result in increased killing by cells from sensitised animals. The effect of opsonising in sera, heat treated at 56°C for 30 min, once again significantly increased phagocytic cell killing of A. fumigatus by approximately 30% ($p<0.05$, Table 3.27). Opsonisation in sera containing specific antibody which had been heat treated for 30 min at 56°C resulted in significantly enhanced killing of A. fumigatus by macrophages both from naive and specifically sensitised animals ($p<0.005$).

Summary

Opsonisation of spores of A.fumigatus in serum containing specific antibody did not significantly increase mouse peritoneal exudate cell killing of A.fumigatus

TABLE 3.27 The effects of opsonisation in naive sera and sera containing specific antibody on the killing of spores of A. fumigatus by macrophages from naive mice and mice sensitised to A. fumigatus incubated for 3 h at 37°C in vitro

Opsonising Sera	Serum Treatment	Macrophage killing of A. fumigatus Cell Source: Naive	Cell Source: Sensitised
Naive	None	32.7 (6.7)[1]	36.4 (6.9)
Naive	56°C 30 min	41.9 (7.1)*	44.7 (6.1)*
Sensitised[2]	None	40.2 (7.4)	36.6 (6.2)
Sensitised	56°C 30 min	52.5 (8.4)**	51.5 (8.7)**

1 % killing of A. fumigatus Mean (SEM)
2 Sera from animals sensitised to A. fumigatus containing specific antibody
* Significantly more killing than untreated serum $p<0.05$
** Significantly more killing than untreated naive serum $p<0.005$

3.13 Examination of the Cell Association and Killing of Fungal Spores by Phagocytic Cells from People with Asthma Compared to Normal Controls

Phagocytic cells were obtained from 42 people evenly distributed between the three clinical groups:

(i) 15 non-asthmatic controls

(ii) 14 asthmatic patients non-sensitised to A. fumigatus

(iii) 13 asthmatic patients sensitised to A. fumigatus

Examination of the ability of monocytes and PMN to handle fungal spores was carried out in order to answer the following questions:

(i) Does the number of spores of A. fumigatus which are bound and killed by human monocytes and PMN differ from that of the control spore P. ochrochloron?

(ii) Is the number of fungal spores killed by monocytes different from that killed by PMN?

(iii) Does opsonisation in heat treated serum have an effect on the phagocytic killing of fungal spores?

(iv) Are there differences between the clinical groups in their phagocytic handling of A. fumigatus, either alone, or compared with P. ochrochloron?

3.13.1 Cell-association and killing of fungal spores by human PMN and monocytes from control subjects

(i) Cell-association

The cell association of fungal spores with both monocytes and PMN was generally very high (>86%). No significant differences between spore type were found. The individual results with mean (SEM) are shown in Fig 3.17.

(ii) Spore killing

Spores of A. fumigatus, opsonised in autologous serum, were significantly more resistant to killing by monocytes ($p<0.025$) and PMN ($p<0.001$) than were similarly opsonised spores of P. ochrochloron. The results are shown in Fig 3.18 and the means (SEM) given in Table 3.28.

3.13.2 A comparison of the handling of fungal spores by monocytes versus PMN from control subjects

Human monocytes were significantly more efficient than PMN at killing spores of A. fumigatus ($p<0.001$) and P. ochrochloron ($p<0.002$) see Table 3.28.

Summary

Although the cell association of the fungal spores to the phagocytic cell was generally very high, spores of A.fumigatus appeared to be more resistant to killing than spores of P.ochrochloron. In addition monocytes were more efficient at killing spores of A.fumigatus and P.ochrochloron than PMN.

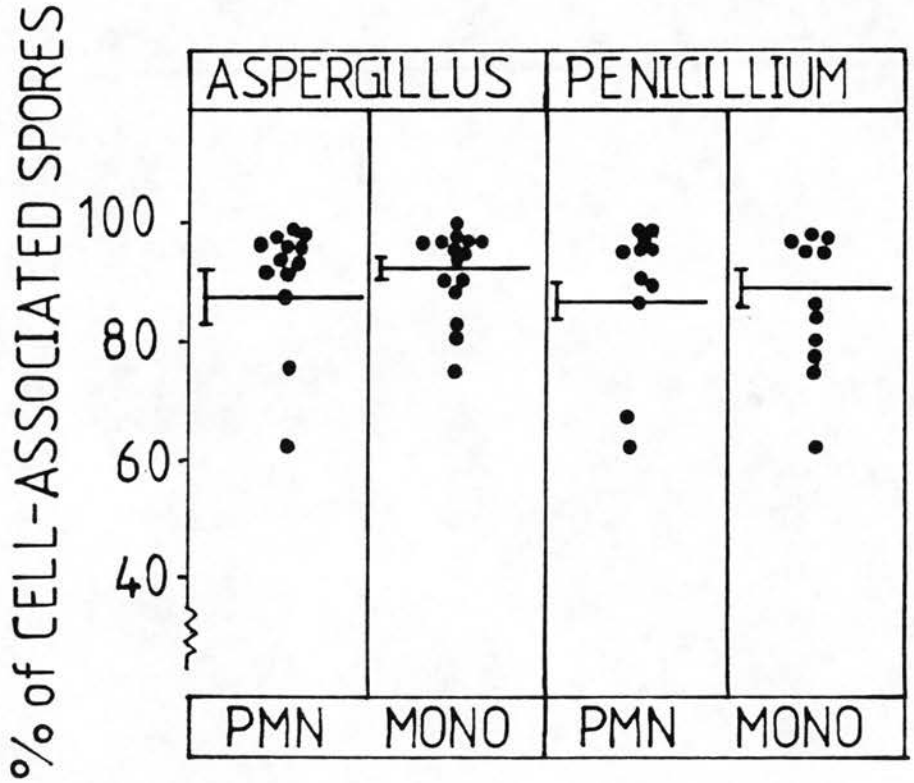


Figure 3.17 The percentage (Mean + SEM) of spores of A. fumigatus and P. ochrochloron, opsonised in 5% autologous serum, becoming cell-associated with human polymorphonuclear leukocytes (PMN) and monocytes (MONO) following incubation in vitro for 1 h at 37°C.

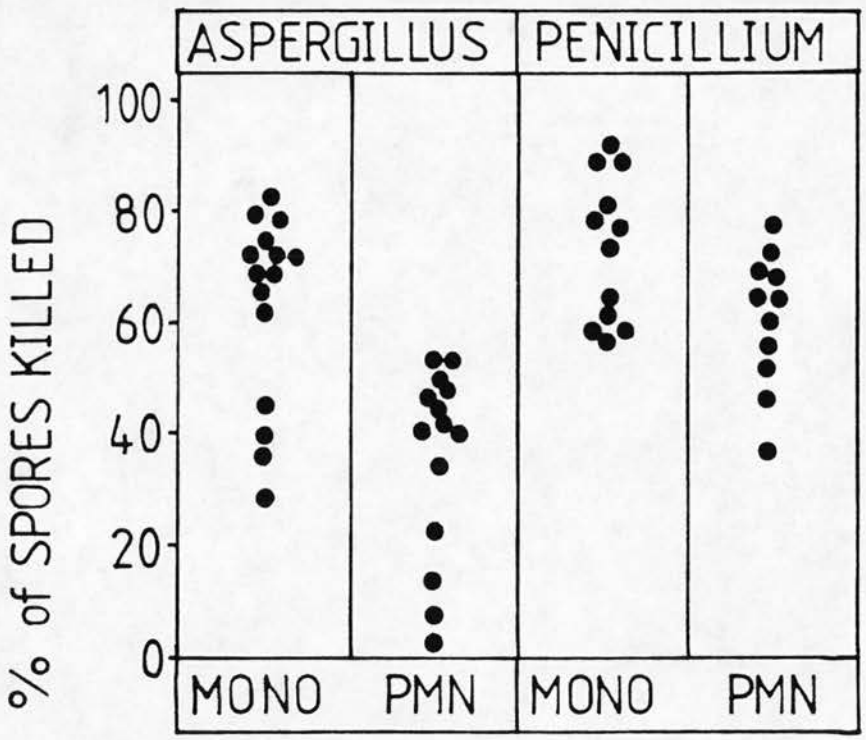


Figure 3.18 The percentage of spores of A. fumigatus and P. ochrochloron, opsonised in 5% autologous serum, which were killed by human monocytes (MONO) and polymorphonuclear leukocytes (PMN) following incubation at 37°C for 3 h.

TABLE 3.28 The percentage killing of spores of *A. fumigatus* and *P. ochrochloron* opsonised in autologous serum by human monocytes and PMN incubated for 3 h at 37°C *in vitro*

Cell	*A. fumigatus*	*P. ochrochloron*
	% killed	
Monocyte	63.3 (4.42)[1]*	73.8 (3.46)
PMN	35.8 (3.87)**	60.5 (3.1)

1 Mean (SEM) of the results from 15 subjects
Significantly less killed than *P. ochrochloron*
* $p<0.025$ ** $p<0.001$

Monocytes significantly more efficient than PMN at killing *A. fumigatus* $p<0.001$ and *P. ochrochloron* $p<0.002$

3.13.3 A comparison of the handling of spores of A. fumigatus and P.ochrochloron by phagocytes from asthmatic patients sensitised and non-sensitised to A. fumigatus versus control subjects

(i) Cell-association

The cell association of monocytes and PMN with spores of A. fumigatus and P. ochrochloron was generally very high. However, fewer spores bound to monocytes and PMN from the control group when compared with binding to cells from asthmatic patients sensitised and non-sensitised to A. fumigatus (Table 3.29).

(ii) Spore killing in autologous serum

People with asthma sensitised to A. fumigatus showed significant differences in their handling of A. fumigatus in vitro when compared with the control group. Monocytes from these patients were significantly less efficient at killing spores of A. fumigatus whilst their PMN were significantly more efficient, when compared with the cells from the control group ($p<0.05$ Table 3.30). Therefore, unlike the results found with the control group no significant differences were found in the killing of A. fumigatus by PMN and monocytes from these patients. The results for the group of non-sensitised asthmatic patients generally fell between those obtained from the control group and the sensitised asthmatic patient group (Table 3.30) except for PMN killing of P. ochrochloron which was decreased.

(iii) Spore killing in heat-treated sera

Opsonisation with sera which had been heated at 56^{o}C for 30 min once again significantly increased the killing of spores of A. fumigatus by monocytes and PMN from all of the patient groups ($p<0.002$, Fig

TABLE 3.29 A comparison of the cell-association of fungal spores opsonised in autologous serum with monocytes and PMN from patients sensitised and non-sensitised to *A. fumigatus*

Spore	Cell	CLINICAL GROUP		
		Control n = 15	Asthmatic Non-Sensitised n = 14	Asthmatic Sensitised n = 13
A. fumigatus	Monocyte	92.3 (1.91)[1]	96.6 (0.8)	96.8 (2.55)
	PMN	87.3 (4.7)	96.1 (0.51)	96.3 (1.4)
P. ochrochloron	Monocyte	86.9 (3.1)	95.0 (1.34)	96.5 (8.4)
	PMN	89.2 (3.3)	93.8 (1.74)	94.7 (3.9)

1 Mean (SEM) of the percentage of spores cell-associated (incubated 1 h at 37°C *in vitro*)

TABLE 3.30 The percentage killing of fungal spores opsonised in autologous serum by monocytes and PMN from patients sensitised and non-sensitised to A. fumigatus

Spore	Cell	CLINICAL GROUP Control n = 15	Asthmatic non-sensitised n = 14	Asthmatic sensitised n = 13
A. fumigatus	Monocyte	63.3 (4.42)[1]	58.9 (4.27)	51.36 (5.05)*
A. fumigatus	PMN	35.8 (3.87)	43.0 (5.1)	51.13 (3.61)*
P. ochrochloron	Monocyte	73.8 (3.46)	74.1 (3.6)	72.70 (3.41)
P. ochrochloron	PMN	60.5 (3.1)	47.0 (3.8)	55.38 (4.28)

1 Mean (SEM) of % of spores killed

* Monocytes kill significantly less and PMN kill significantly more A. fumigatus when compared with the control group $p<0.05$

3.19). No such effect was found for P. ochrochloron; the means (SEM) are given in Table 3.31.

(iv) Comparison between the killing of fungal spores by asthmatic patients sensitised to A. fumigatus subdivided into two clinical groups

To determine what contributions the cells and serum from patients diagnosed as having allergic bronchopulmonary aspergillosis may have to these findings, the group of asthmatic patients sensitised to A. fumigatus (n = 13) was divided into two subgroups (i) simple asthmatics sensitised to A. fumigatus (n = 5), (ii) patients diagnosed as having allergic bronchopulmonary aspergillosis (n = 8). The comparison between these two groups with regard to the phagocytic killing of spores of A. fumigatus and P. ochrochloron, opsonised in autologous and heat-treated sera was made. The results illustrated in Fig 3.20 show that there were no differences between the two groups in the killing of A. fumigatus. Although there appear to be some differences between the groups in the killing of P. ochrochloron, especially after opsonisation in heat treated serum, they are not statistically significant. The analyses of variance Table is given in the Appendix (Table A10).

Summary

Patients sensitised to A. fumigatus showed significant differences in the ability of their cells to kill spores of A. fumigatus which had been opsonised in autologous serum. However, these differences were not evident when the spores were opsonised in serum (autologous) which had been heat-treated as this resulted in an overall increase in the phagocytic cell killing of A. fumigatus. No such differences were

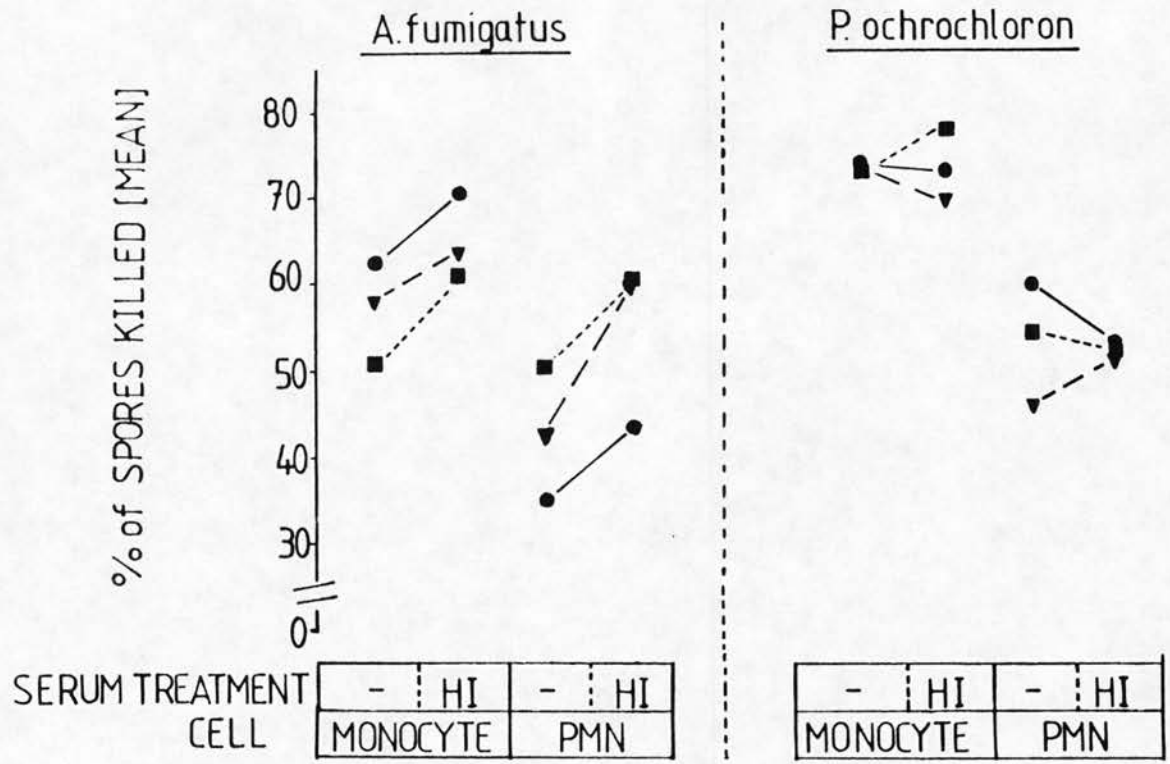


Figure 3.19 A comparison of the ability of monocytes and PMN, from patients in the three clinical groups to kill spores of A. fumigatus and P. ochrochloron following incubation at 37°C for 3 h. The spores were opsonised in 5% autologous serum which had been untreated (——) or heat-treated for 30 min at 56°C (HI). Controls, n = 15 (●——●) asthmatic patients sensitised to A. fumigatus, n = 13 (■- - -■), asthmatic patients non-sensitised, n = 14 (▼·—·▼). The results are expressed as mean percentages.

Opsonisation of spores of A. fumigatus in heat-treated serum significantly increased killing of A. fumigatus by PMN and monocytes from all patient groups $p<0.002$

TABLE 3.31 The percentage killing of fungal spores opsonised in autologous serum with and without heat treatment by monocytes and PMN from patients sensitised and non-sensitised to A. fumigatus

Spore	Cell	Serum Treatment	CLINICAL GROUP		
			Control n = 15	Asthmatic non-Sensitised n = 14	Asthmatic Sensitised n = 13
A. fumigatus	Monocyte	Untreated	63.3 (4.42)[1]	58.9 (4.27)	51.36 (5.05)
" "		56°C 30 min	71.1 (3.20)*	64.7 (5.2)*	61.85 (5.72)*
" "	PMN	Untreated	35.8 (3.87)	43.0 (5.1)	51.13 (3.61)
" "		56°C 30 min	44.0 (2.12)*	60.1 (4.73)*	61.14 (2.05)*
P. ochrochloron	Monocyte	Untreated	73.8 (3.46)	74.1 (3.6)	72.70 (3.41)
" "		56°C 30 min	73.8 (3.92)	70.1 (3.16)	79.09 (3.01)
" "	PMN	Untreated	60.5 (3.1)	47.0 (3.8)	55.38 (4.28)
" "		56°C 30 min	54.2 (2.35)	52.2 (4.14)	52.9 (5.25)

1 Mean (SEM) of % of spores killed

* Significantly more killing of A. fumigatus opsonised in heat-treated serum (56°C 30 min) than untreated serum $p<0.002$

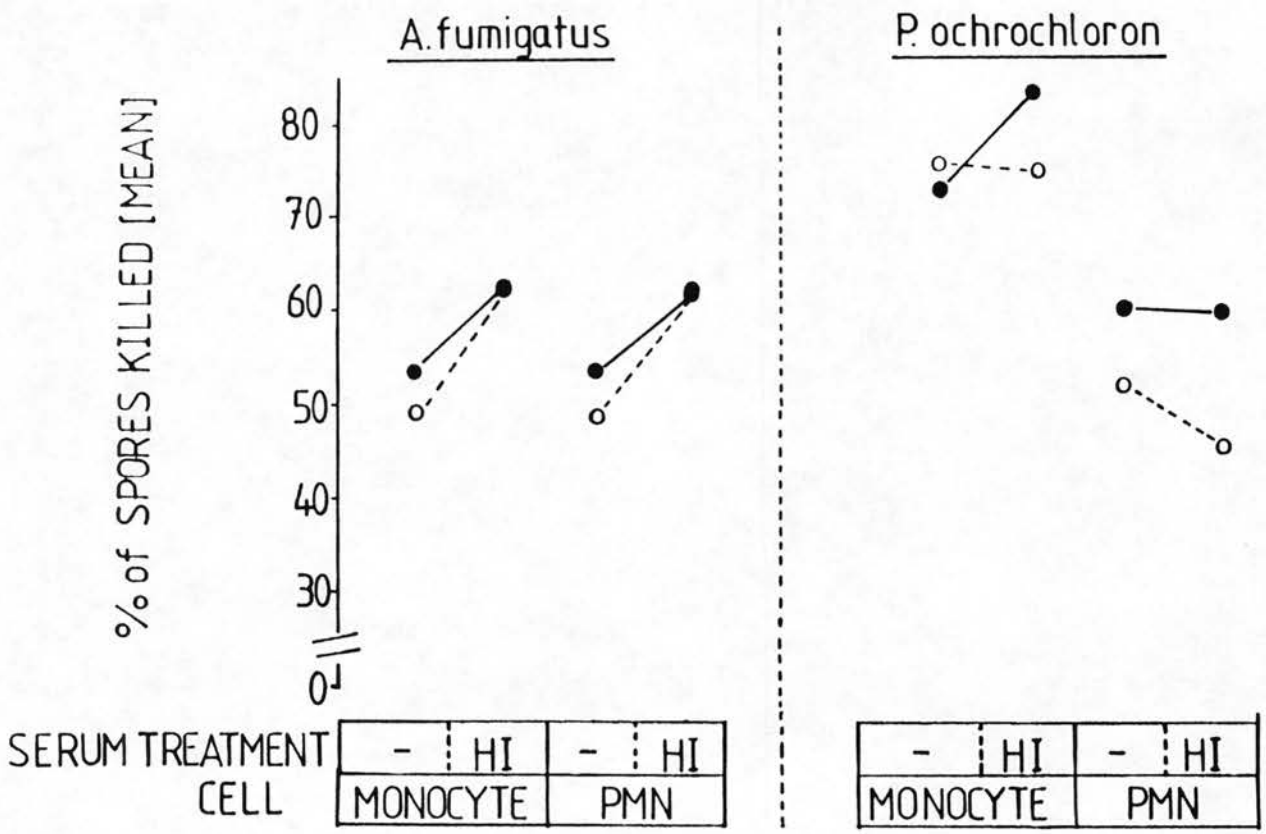


Figure 3.20 A comparison of the percentages of spores of A. fumigatus and P. ochrochloron killed by monocytes and PMN from the asthmatic patients sensitised to A. fumigatus who had been subdivided into two groups:- (i) simple asthmatics sensitised to A. fumigatus n = 5 (O----O) (ii) patients diagnosed as having allergic bronchopulmonary aspergillosis n = 8 (●——●). The spores were opsonised in 5% autologous serum which had been untreated (—) or heat-treated for 30 min at 56°C (HI). Results expressed as mean percentages.

found for spores of P. ochrochloron.

3.14 Effect of Corticosteroid Treatment on the Phagocytic Killing of Fungal Spores

To determine if corticosteroid treatment affected phagocytic cell-association and killing of fungal spores, patients from the asthmatic group sensitised to A. fumigatus were again subdivided into two groups (i) patients not on corticosteroids (n = 7), (ii) patients on corticosteroid treatment (n = 6); three on intramuscular triamcinolone acetonide - two on 40 mg per month, one on 60 mg per month: the other three on oral prednisolone 10 mg per day.

The results in Table 3.32 show that corticosteroid treatment had no effect on the association of fungal spores with phagocytic cells. No differences between the two groups were found in the ability of phagocytic cells to kill spores of A. fumigatus opsonised in untreated serum. Although patients on corticosteroids appeared to show increased killing of spores of P. ochrochloron, it was not statistically significant (Table 3.33). A comparison between opsonising in serum with and without heat treatment is illustrated in Fig 3.21. The analysis of variance Table is given in the Appendix, Table A11.

TABLE 3.32 The effect of corticosteroid treatment on the cell-association of fungal spores with monocyte cells from patients with asthma sensitised to *A. fumigatus*

Spore	Cell	Patient Treatment Non-steroid n = 7	Steroid n = 6
A. fumigatus	Monocyte	95.7 (3.1)[1]	98.0 (1.4)
	PMN	96.0 (1.5)	96.7 (2.1)
P. ochrochloron	Monocyte	95.7 (2.9)	97.1 (1.9)
	PMN	94.3 (4.4)	94.8 (3.9)

1 % of spore becoming cell-associated - Mean (SD) after 1 h at 37°C *in vitro*

TABLE 3.33 The effect of corticosteroid treatment on the killing of fungal spores by monocytes and PMN from patients with asthma sensitised to A. fumigatus

Spore	Cell	Opsonin Autologous Serum	Patient Treatment	
			Non-steroid n = 7	Steroid n = 6
A. fumigatus	Monocyte	untreated	51.8 (19.6)[1]	51.0 (18.3)
"	Monocyte	HI 56°C	62.7 (22.5)	61.0 (21.0)
"	PMN	untreated	50.2 (13.8)	52.1 (7.8)
"	PMN	HI 56°C	60.3 (10.0)	62.0 (3.6)
P. ochrochloron	Monocyte	untreated	68.3 (16.0)	76.3 (6.8)
"	Monocyte	HI 56°C	76.7 (12.3)	80.4 (10.7)
"	PMN	untreated	55.5 (18.8)	56.6 (14.1)
"	PMN	HI 56°C	48.6 (18.5)	57.0 (20.4)

1 % of spores killed - mean (SD)

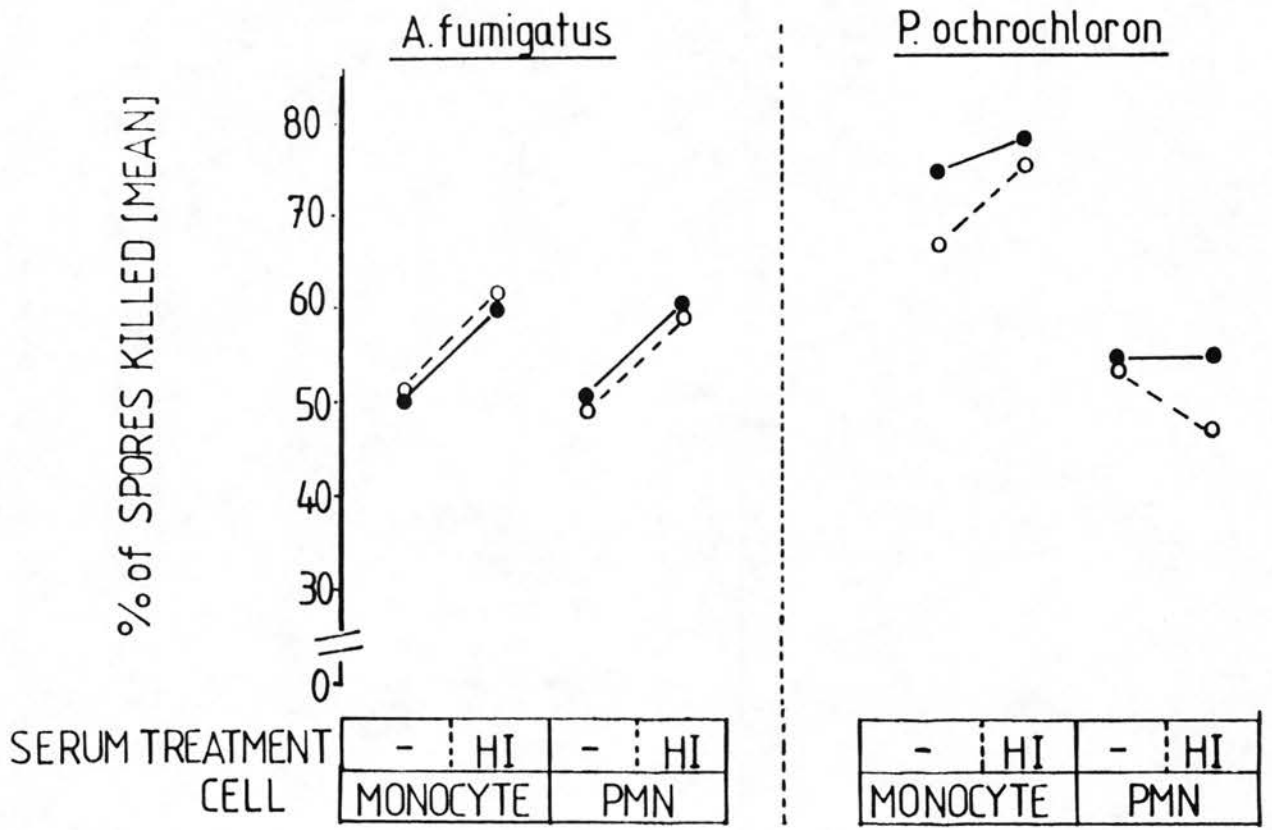


Figure 3.21 The effect of corticosteroid treatment on the killing of spores of A. fumigatus and P. ochrochloron by monocytes and PMN from asthmatic patients sensitised to A. fumigatus subdivided into two groups:- (i) patients not on corticosteroids n = 7 (O---O), (ii) patients on corticosteroids n = 6 (●——●). The spores were opsonised in 5% autologous serum which had been untreated (—) or heat-treated for 30 min at 56°C (HI). Results expressed as mean percentages.

3.15 Comparison between Clinical Groups on the Production of Reactive Oxygen Intermediates by Phagocytic Cells in Response to Fungal Spores

3.15.1 Chemiluminescence

Blood was taken from a total of 16 people who were subdivided into the three clinical groups:

(i) Five non-asthmatic controls

(ii) Five with asthma but not sensitised to A. fumigatus

(iii) Six with asthma sensitised to A. fumigatus

Chemiluminescent responses to monocytes and PMN was assessed to determine if there were any differences between the clinical groups. No significant differences were found between clinical groups in the chemiluminescent responses of monocytes and PMN to spores of A. fumigatus and P. ochrochloron opsonised in 5% autologous serum (illustrated in Fig 3.22).

Opsonisation in autologous serum (when compared to control AB serum,) increase the chemiluminescent responses to P. ochrochloron of both monocytes and PMN from patients with asthma sensitised to A. fumigatus, by approximately 45% as opposed to the 20% increase found with the control group (Table 3.34).

Opsonisation of A. fumigatus in serum which was heat-treated (56^{o} 30 min) had no effect on the chemiluminescent responses of PMN from asthmatic patients sensitised to A. fumigatus; whilst the PMN from the control group showed a significant decrease $p<0.05$, see Table 3.35. The percentage effect of heat inactivation on the chemilum-

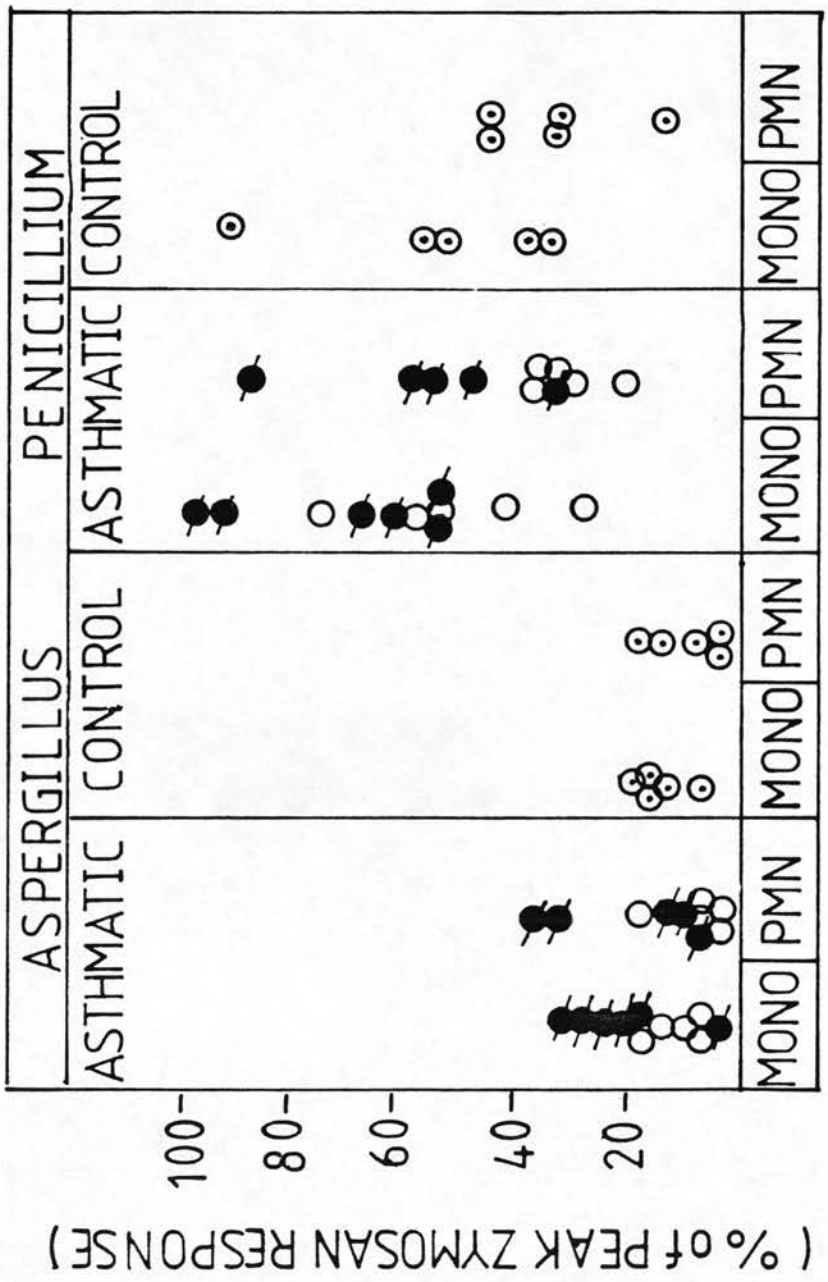


Figure 3.22 Chemiluminescent responses of monocytes (Mono) and PMN, from patients in the three clinical groups, to spores of A. fumigatus and P. ochrochloron, opsonised in 5% autologous serum. Asthmatic patients non-sensitised to A. fumigatus (O), asthmatic patients sensitised to A. fumigatus (●), control subjects (⊙). Results shown are the peak chemiluminescent responses for each subject expressed as a percentage of the zymosan response.

TABLE 3.34 Effect of opsonisation of fungal spores in autologous versus AB serum on the chemiluminescent responses of monocytes and PMN from people sensitised and non-sensitised to A. fumigatus

Spore	Cell	Serum	Control	CLINICAL GROUP Asthma Non-sensitised	Asthma Sensitised
A. fumigatus	Monocyte	AB	14 (1.2)[1]	9 (1.2)	18 (1.2)[2]
	Monocyte	autologous	15 (1.2)	10 (1.2)	18 (1.3)[2]
P. ochrochloron	Monocyte	AB	46 (1.3)	45 (1.2)	46 (1.3)[2]
	Monocyte	autologous	56 (1.2)	49 (1.2)	65 (1.1)
A. fumigatus	PMN	AB	7 (1.4)	5 (1.5)	13 (1.3)
	PMN	autologous	9 (1.5)	6 (1.3)	15 (1.4)
P. ochrochloron	PMN	AB	28 (1.3)	26 (1.2)	36 (1.4)
	PMN	autologous	34 (1.3)	30 (1.1)	55 (1.2)

1 Geometric mean (SEM) of the chemiluminescent response expressed as a % of zymosan
2 The number of subjects in the group is 6. All the other results are the means of 5 subjects

TABLE 3.35 The effect of spore opsonisation in complete versus heat-treated serum on the chemiluminescent responses of monocytes and PMN from people sensitised and non-sensitised to A. fumigatus

Spore	Cell	Serum Treatment	CLINICAL GROUP		
			Control	Asthma Non-sensitised	Asthma Sensitised
			Peak chemiluminescence (mV)		
A. fumigatus	Monocyte	None	40.2 (1.25)[1]	11.9 (1.35)	18.9 (1.29)
	Monocyte	50°C 20 min	20.4 (1.34)	7.1 (1.33)	12.7 (1.23)
	Monocyte	56°C 30 min	12.8 (1.39)	5.5 (1.40)	7.8 (1.41)
P. ochrochloron	Monocyte	None	104.9 (1.35)	50.8 (1.62)	47.3 (1.39)
	Monocyte	50°C 20 min	68.6 (1.27)	28.1 (1.54)	27.4 (1.26)
	Monocyte	56° 20 min	35.1 (1.35)	14.7 (1.69)	18.4 (1.53)
A. fumigatus	PMN	None	72.6 (1.22)	30.0 (1.55)	35.5 (1.85)
	PMN	50°C 20 min	64.4 (1.26)	38.5 (1.53)	33.0 (1.93)
	PMN	56°C 30 min	49.2 (1.28)	31.1 (1.56)	30.8 (1.86)*
P. ochrochloron	PMN	None	217.7 (1.25)	105.4 (1.83)	88.2 (2.27)
	PMN	50°C 20 min	143.3 (1.22)	86.0 (1.96)	72.2 (2.21)
	PMN	56°C 30 min	100.9 (1.27)	60.2 (1.92)	44.9 (2.02)

1 Geometric mean (SEM)

* No real decrease in chemiluminescence following opsonisation in heat treated serum versus untreated serum. This is significantly different from the results of the control group which do show a significant decrease from untreated serum $p<0.05$

inescent responses is illustrated in Fig 3.23. The analyses of variance Table for this work is given in the Appendix (Table A1). The effect of specific antibody was not examined using this assay system as only one of the patients showed evidence of precipitating antibodies.

3.15.2 Superoxide anion

Phagocytic cells were obtained from a total of 26 people who were subdivided between the clinical groups as follows:

(i) 5 non-allergic controls

(ii) 10 with asthma non-sensitised to A. fumigatus

(iii) 11 with asthma sensitised to A. fumigatus - as well as positive Type I skin test reactions. Ten of these patients had either precipitating antibodies and/or specific IgE antibody to A. fumigatus

A comparison between patient groups in the amount of superoxide anion produced by phagocytic cells in response to spores of A. fumigatus, spores of P. ochrochloron and zymosan was measured. In addition the effects of opsonisation on the production of superoxide anion by phagocytic cells in response to A. fumigatus was estimated.

No significant differences in the production of superoxide anion by phagocytic cells were found between clinical groups (Table 3.36). Even although the spontaneous release of superoxide anion by monocytes in the two groups of patients with asthma, appeared to be increased when compared with the control group; it was not statistically significant (probably because of the high SD). The analyses

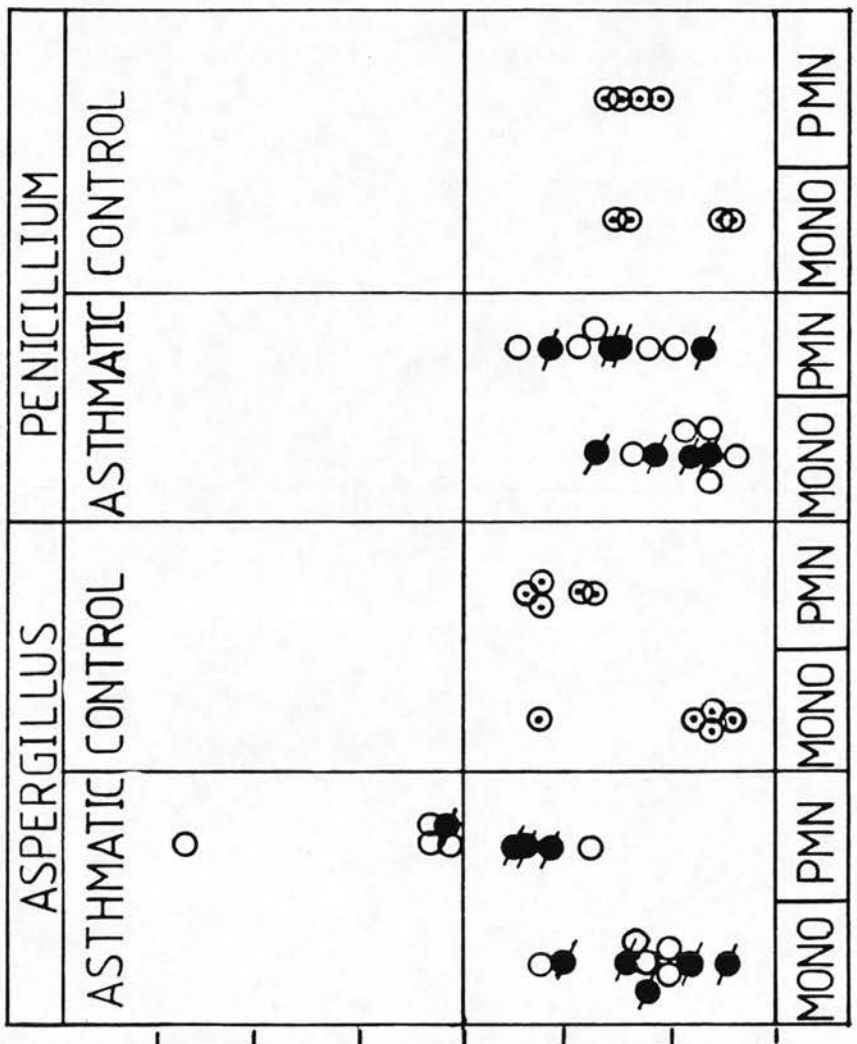


Figure 3.23 Effect of heat-treating the opsonising sera (56°C 30 min) on the chemiluminescent responses of monocytes (MONO) and PMN, from patients in the three clinical groups, to spores of A. fumigatus and P. ochrochloron. Asthmatic patients sensitised to A. fumigatus (●), asthmatic patients non-sensitised (○), control subjects (⊙). Results expressed as the percentage change in the peak chemiluminescent response following opsonisation in 5% heat-treated sera when compared with that obtained using 5% untreated sera.

TABLE 3.36 Superoxide anion production by monocytes and PMN, from patients in the three clinical groups and spontaneous release (HBSS) in response to zymosan spores of P. ochrochloron and A. fumigatus opsonised in AB serum

Cell	Trigger	CLINICAL GROUP: Control	Asthma Non-sensitised n = 10	Asthma sensitised n = 11
		Superoxide anion nmol/5 x 10^5 cells/2 h		
Monocyte	HBSS[1]	6.46 (1.7)[2]	9.01 (5.01)	9.11 (4.95)
"	Zymosan	13.94 (2.9)	15.57 (3.0)	14.53 (3.4)
"	P. ochrochloron	6.18 (2.6)	7.01 (4.6)	6.02 (3.5)
"	A. fumigatus	4.8 (1.3)	5.74 (3.7)	5.18 (2.01)
PMN	HBSS	13.6 (4.4)	13.41 (6.8)	10.72 (3.29)
"	Zymosan	15.53 (4.4)	15.28 (3.9)	15.12 (5.59)
"	P. ochrochloron	13.30 (3.9)	11.64 (5.3)	11.40 (3.4)
"	A. fumigatus	9.9 (2.25)	8.87 (3.8)	9.37 (1.6)

1 Hanks' balanced salt solution

2 Mean (SD)

of variance Table is given in the Appendix (Table A4).

There were no differences in the amount of superoxide anion released, in response to spores of A. fumigatus, opsonised in AB sera compared with autologous sera. Similarly opsonisation of A. fumigatus in autologous sera which had been heat-treated at 56°C for 30 min had no effect (see Table 3.37).

Therefore, opsonisation in specific antibody did not affect the release of superoxide anion by phagocytic cells. In addition opsonisation in heat treated serum did not alter the amount of superoxide anion produced in response to spores of A. fumigatus.

Summary

The principal conclusions arising from comparison between the three clinical groups are that cells from asthmatic patients, sensitised to A. fumigatus, show significant differences in their ability to kill spores of A. fumigatus opsonised in autologous serum. No such differences were found for spores of P. ochrochloron. Corticosteroid treatment (maintenance dose) had no effect on fungal killing by phagocytic cells. Phagocytic cells from all three clinical groups failed to elicit a 'respiratory burst' to spores of A. fumigatus even when opsonised in specific antibody.

TABLE 3.37 Superoxide anion production by monocytes and PMN, from patients in the three clinical groups in response to spores of A. fumigatus opsonised in AB serum and autologous serum untreated and heat-treated

Cell	Opsonising	CLINICAL GROUP Control n = 5	Asthma Non-sensitised n = 10	Asthma Sensitised n = 11
		Superoxide anion nmol/5 x 10^5 cells/2h		
Monocyte	AB	4.8 (1.3)[1]	5.74 (3.7)	5.18 (2.09)
	Autologous	4.5 (1.1)	5.53 (3.6)	5.13 (2.25)
	Auto HI56	4.1 (1.5)	5.30 (2.6)	5.12 (3.31)
PMN	AB	9.9 (2.25)	8.87 (3.8)	9.37 (1.6)
	Autologous	10.04 (2.13)	8.90 (3.4)	9.49 (1.8)
	Auto HI56	10.16 (1.37)	8.96 (3.6)	9.67 (1.9)

1 Mean SD

3.16 Principal Findings

1. Although a substantial number of spores of *A. fumigatus* become cell-associated the majority remain on the surface of the cell without becoming ingested.

2. Opsonised spores of *A. fumigatus* fail to elicit a 'respiratory burst' from phagocytic cells.

3. Spores of *A. fumigatus* release a substance which, although not cytotoxic, interferes with phagocytic cell function including phagocytosis, production of reactive oxygen intermediates, chemotaxis and spreading.

4. The inhibitory component of the spore diffusate is released immediately the spores are put into solution, and has a molecular weight of between 1,000 and 5,000 daltons. The exact biochemical nature of the substance has still to be established.

5. Spores of *A. fumigatus*, opsonised in autologous serum are significantly more resistant to killing by phagocytic cells from humans and rodents than similarly opsonised spores of *P. ochrochloron*.

6. Serum contains a heat labile component which inhibits phagocytic cell killing of spores of *A. fumigatus*. No such effect was found with spores of *P. ochrochloron*.

7. Human monocytes are more efficient than PMN at killing fungal spores.

8. Comparisons between the three clinical groups showed that patients sensitised to A. fumigatus gave significant differences in the ability of their phagocytic cells to kill spores of A. fumigatus, opsonised in autologous serum, when compared with the control group.

9. The presence of specific antibody in the opsonising serum did not significantly increase the phagocytic killing of A. fumigatus.

10 Corticosteroid treatment (maintenance dose) had no effect on fungal killing by phagocytic cells.

4. DISCUSSION

What is so special about A. fumigatus as opposed to many other air spora which enables it to cause ill-health in man and animals? An intriguing aspect of the biological behaviour of A. fumigatus is its ability to initiate so many disease processes. Thus it may, depending on the appropriate circumstances, provoke asthma, extrinsic allergic alveolitis, allergic bronchopulmonary aspergillosis, form into a mycetoma or act as a true opportunistic pathogen by becoming invasive[2,3].

Mullins and Seaton in 1978 found that A. fumigatus could be isolated from post mortem human lung specimens more frequently than would have been anticipated from the prevalence of its spores in the air. This led to the suggestion that as well as having an optimum growth temperature of 37°C and a spore size which enabled it to penetrate deep into the lung the fungus may also be particularly resistant to the host defence network[152]. This hypothesis was the starting point of this Thesis, leading to an investigation of phagocytic cells, a key component of the network. Though clearly important in host defences against invading microorganisms, the precise role of these cells in the handling of A. fumigatus remained undefined.

4.1 Phagocytosis

Phagocytosis is often an important step in the killing of most microorganisms and it has often been assumed that spores of A. fumigatus are phagocytosed prior to becoming killed[221,223]. However, the

results of this study have shown that spores of A. fumigatus are relatively resistant to phagocytosis by human and mouse phagocytes. Whilst initial studies using light microscopy did not allow differentiation of ingested and surface-bound spores, further work using Nomarski optics revealed that a substantial number of spores of A. fumigatus appeared to be on the surface of the cell; in contrast spores of P. ochrochloron appeared to be phagocytosed more readily although they also were seen, in smaller numbers, on the cell surface. These observations proved difficult to quantitate but were supported by further observations using scanning electron microscopy, which showed that a high proportion of spores of A. fumigatus (and a lower proportion of spores of P. ochrochloron) were not taken into the cell, remaining bound to the surface. These findings appeared not to be artefacts of in vitro experimentation since similar results were obtained when spores were injected directly into peritoneal cavities of mice and the cells subsequently removed by lavage.

Since these observations were subjective attempts were made to quantify the ratio of extracellular to intracellular spores by means of enzyme stripping technique, but it proved very difficult to remove spores from the cell surfaces. Further efforts, using transmission electron microscopy, also failed, as it proved impossible to differentiate between ingested spores and those sectioned between folds of the phagocyte cell membrane. The difficulties involved in discriminating between attached and ingested particles have previously been discussed by van Furth and Diesselhoff-den Dulk who stated that:

"Even the application of electron microscopy to thin sections cannot solve this problem, because particles trapped between folds of the cell membrane can seem in tangential sections to lie inside the cell"[257].

In spite of this, Levitz and Diamond, using transmission electron microscopy of peroxidase-stained sections of spores of A. fumigatus which had been incubated with PMN at a spore:cell ratio of 10:1 in PBS for 30 min, apparently found that phagosome-lysosome fusion had occurred and that >95% of the cell-associated spores of A. fumigatus were intracellular; it is difficult to assess the validity of these findings since no examples of this work were actually given in the paper[258]. In contrast the suggestion that spores of A. fumigatus may be resistant to phagocytosis is supported by the work of Kurup who, using rabbit alveolar macrophages in vitro could show no evidence of phagosome-lysosome fusion following phagocytosis of A. fumigatus[227]. This may also be the explanation of the finding of Lehrer and Jan who showed that apparently phagocytosed spores (as judged by light microscopy) were nevertheless resistant to killing[226].

4.2 'Respiratory burst'

An important feature of the microbicidal action of phagocytic cells is their capacity to undergo a 'respiratory burst'[201]. This can be detected by measuring the amount of chemiluminescence or specific reactive oxygen intermediates (for example superoxide anion and hydrogen peroxide) produced by phagocytic cells following challenge with microorganisms in vitro.

In addition to their apparent resistance to ingestion, spores of A. fumigatus also failed to induce a significant respiratory burst by human and rodent phagocytic cells. The chemiluminescence produced in response to spores of A. fumigatus was minimal when compared with that obtained following challenge with either zymosan or spores of P. ochrochloron.

When the specific reactive oxygen intermediates superoxide anion and hydrogen peroxide were measured, using phagocytic cells from C. parvum-stimulated mice, it was found that spores of A. fumigatus failed to trigger an increase in superoxide anion release while both zymosan and spores of P. ochrochloron triggered a substantial increase. Similarly, the amount of superoxide anion produced by human PMN and monocytes in response to spores of A. fumigatus was significantly less than that produced in response to zymosan or spores of P. ochrochloron; indeed even opsonisation in specific antibody had no effect. This suggested that spores of A. fumigatus were failing to trigger the primary component of the respiratory burst.

The normal sequence of events in release of reactive oxygen intermediates by phagocytic cells is the production of superoxide anion, which can then either spontaneously or with the help of superoxide dismutase form hydrogen peroxide[259]. If spores of A. fumigatus fail to trigger an increased release of detectable amounts of superoxide anion, it might be expected that hydrogen peroxide levels would also be low, unless rapid dismutation of superoxide anion was occurring. Spores of A. fumigatus as well as failing to trigger superoxide anion release also, at a spore:cell ratio of 25:1 and above, suppressed

hydrogen peroxide production by C. parvum stimulated mouse peritoneal exudate cells when compared with either spores of P. ochrochloron or zymosan. The response of stimulated peritoneal exudate cells to triggering with zymosan, by releasing increased amounts of superoxide anion without an increase in the release of hydrogen peroxide has been reported previously both from our laboratory[260] and by Karnovsky and Badwey[261]. Apart from the slight decrease found in the production of superoxide anion by human monocytes, the release of reactive oxygen intermediates in response to spores of the control fungus P. ochrochloron generally paralleled those released in response to zymosan. A similar trend has also recently been reported by Levitz and Diamond who found that spores of A. fumigatus induce human PMN to release significantly less superoxide anion, hydrogen peroxide, and hypochlorous acid than do Candida albicans or zymosan[258]. However, the amount of superoxide anion generated by PMN in response to spores of A. fumigatus in that study was still significantly more than the spontaneous release by PMN alone (A. fumigatus 15 nmol, spontaneous release 1 nmol). Although they used the same basic method of measuring superoxide anion as that used in this Thesis, namely, determination of the portion of reduced cytochrome C which was inhibitable by superoxide dismutase, the differences between the two studies in the amount of superoxide anion produced either spontaneously or in response to spores of A. fumigatus may possibly be accounted for by different times and methods of incubation. It is known that the phagocytic cell 'respiratory burst' can be induced following the pertubation of the membrane which occurs when the cells come into contact with 'foreign' substances[201,262]. Chari-Bitron et al who followed the uptake of opsonised zymosan by alveolar macrophages,

found that the intensity of chemiluminescence was related to the number of particles becoming attached, both reaching a peak at the same time[263]. Levitz and Diamond incubated their cells/spores for 10 minutes in a shaking water bath, the continual mixing of spores with cells would result in constant membrane pertubation of the cell by the particle thereby increasing non-specific release of superoxide anion. This was indicated by their results which showed[258] that the combination of specific and non-specific triggering by zymosan using the shaking method induced the PMN to release three times the amount (45.1 nmol) of superoxide anion in 10 minutes, compared with the amount (15.5 nmol) produced by PMN after a two hour incubation with no shaking in the present study. It may be that if Levitz and Diamond[258] had not shaken the cells with the spores they would have found less superoxide anion produced in response to A. fumigatus.

It was curious, therefore, that even though a high percentage of spores of A. fumigatus were becoming attached and presumably inducing membrane pertubation, this was not sufficient to elicit a significant 'respiratory burst.

4.3 Spore Diffusates of A. fumigatus

The work discussed above led to the hypothesis that spores of A. fumigatus may produce a substance which interferes with the production of reactive oxygen intermediates and phagocytosis. Studies carried out to test this hypothesis showed that diffusates from spores of A. fumigatus significantly suppressed the spontaneous release of reactive oxygen intermediates by C. parvum-elicited rodent

phagocytic cells and that this inhibitory effect was dose-dependent. No corresponding effect was found with diffusates from spores of P. ochrochloron. The inhibitory component of the spore diffusate of A. fumigatus was released immediately the spores were put into suspension and continued to be released after the spores had been washed. The spore diffusates were shown to be affecting the production of reactive oxygen intermediates and not simply scavenging them once they had been produced. In addition it was confirmed using two separate assay systems (trypan blue exclusion[237] and ^{51}Cr release[255]) that the diffusates were not cytotoxic.

Further investigations of the effects of the diffusate from A. fumigatus spores using an assay of quantitative phagocytosis[245] showed that the diffusates had a significant inhibitory effect: diffusates from P. ochrochloron had no such activity. This observation which supported the earlier microscopical findings is consistent with a recent report by Mullbacher et al who found that gliotoxin isolated from 3-day culture supernatants of A. fumigatus had an inhibitory effect on the phagocytosis of carbon particles by mouse peritoneal exudate cells[264]. This same group in another study just published have also shown that gliotoxin inhibited the basal rate of hydrogen peroxide production by human PMN. However, unlike the results reported in this Thesis using spore diffusates of A. fumigatus, gliotoxin could not inhibit phorbol myristate acetate triggered production of hydrogen peroxide[265]. The difference between gliotoxin and the diffusate described in the present study is that gliotoxin could not be isolated until the spores had been in culture for at least three days, at which time mycelial growth would

be expected to have been abundant. In contrast the diffusate described here diffuses from the respirable sized spores as soon as they are put into suspension and its quick release suggests that it is a constituent present on or close to the surface of the spore rather than a metabolite which is produced as a result of germination. The inhibitory component of the diffusate has a molecular weight of between 1,000 and 5,000 daltons and preliminary biochemical analysis indicates that it is an aromatic compound.

In an attempt to pinpoint the effector mechanism of the inhibitory component of the spore diffusate of A. fumigatus, its effect on cell movement, a fundamental aspect of phagocytic cell activation, was examined. In order for a phagocytic cell to become activated thereby enhancing its phagocytic ability, a rearrangement of the cell membrane must occur[207,266]. This involves a complex process which requires a co-ordinated interaction of the plasma membrane with contractile elements in the cytoplasm[267,268]. The changes are reflected in the enhanced ability of the cell to spread over a surface and to migrate in response to chemical stimuli. Such functions are readily measured in vitro, and it was found that both these aspects of phagocyte activity were inhibited by spore diffusates of A. fumigatus. In contrast, spores diffusates of P. ochrochloron had no effect.

Thus it appears that A. fumigatus spores release a diffusable substance that has several effects of considerable biological importance in ensuring their survival in the lung and therefore enhancing their pathogenic potential. Possibly the diffusate is the first mechanism

by which the spore reduces the efficiency of the phagocyte while gliotoxin, which is produced once germination has taken place, may be a second line of anti-phagocytic defence used by A. fumigatus to establish itself and remain within the lung.

The actions of the A. fumigatus diffusate appear to be similar to those of a group of fungal metabolites called cytochalasins (Greek: cytos-a cell, chalasis-relaxation)[269,270]. Cytochalasin B, the most extensively studied of these metabolites, has a molecular weight of less than 500 daltons and has been shown to inhibit phagocytosis, cell spreading and chemotaxis[271,272] and like the diffusate, is not directly toxic to the cells. Cytochalasin B has been shown to act by affecting the orientated microfilaments of the substratum-associated regions of the plasma membrane[273]. It also affects re-ordering of the lipid membrane causing depolarisation of the plasma membrane[274,275]. The region of the plasma membrane on which the spore diffusate of A. fumigatus exerts its influence has still to be established. Other studies on A. fumigatus have shown that a water soluble substance (molecular weight >10,000 daltons), obtained from seven day cultures, could interfere with complement activation[276], whilst Chaparas et al found that three day-old culture supernatants of A. fumigatus could inhibit mitogen-induced lymphocytic transformation[277].

It is perhaps important to remember at this point that the pathogenicity of A. fumigatus should be regarded as incidental to its normal saprophytic life cycle in nature[278]. These biological properties of the fungus may have evolved as a defence mechanism in its

natural habitat, the soil, in order to protect itself against phagocytosis by its natural enemies, the amoebae. Support for this hypothesis comes from the work of Old and Derbyshire who, whilst conducting feeding trials to assess the ability of the giant vampyrellid amoeba (Arachnula impatiens cienk), to attach and lyse fungal spores, found that spores of a few fungal species were resistant to ingestion; among those was A. niger[279].

4.4 Killing of A. fumigatus Spores

These investigations lead to the conclusion that spores of A. fumigatus are able to resist phagocytosis at least in part by interfering with cell membrane movement. The biological relevance of this anti-phagocytic property was assessed by looking at the ability of phagocytic cells to kill spores of A. fumigatus as compared to the control spores of P. ochrochloron. Again spores of A. fumigatus proved more resistant to killing than did spores of P. ochrochloron, whether the phagocytic cells were mouse peritoneal macrophages, rat alveolar macrophages, human peripheral blood monocytes and PMN, or human pulmonary macrophages. The percentage killing of A. fumigatus ranged from 17% with rat alveolar macrophages to 63% with human monocytes, but in all experiments spores of A. fumigatus were more resistant to killing than were spores of P. ochrochloron.

Although there are difficulties in extrapolating the findings of this study to those of others because of differences in experimental procedure, such as spore:cell ratios, incubation periods and methods

of estimating germination, a reasonable amount of agreement can be found. The percentage killing of A. fumigatus found using rat alveolar macrophages was similar to that found by Kurup who used rabbit alveolar macrophages[227] and by Waldorf who used mouse alveolar macrophages[228]. Although human alveolar macrophages appear to be more efficient at killing spores of A. fumigatus than do the rat alveolar macrophages this may be related to differences in their state of functional activation. The alveolar macrophages from rats used in the work reported in this Thesis were obtained from specific pathogen-free animals and were usually in a resting state whereas the human pulmonary macrophages were obtained from lungs of people (mainly city-dwellers who would have been constantly exposed to normal environmental agents) and would perhaps have been more activated. Previous studies have shown PVG rat alveolar macrophages which had been activated by quartz inhalation to be more efficient at killing spores of A. fumigatus than alveolar macrophages from the non-stimulated animals[280]. The levels of killing of A. fumigatus spores by mouse peritoneal exudate cells in the present study are substantially higher than those obtained by Schaffner et al[222]. These authors used CF1 mice, whilst the results reported in this Thesis were obtained using C57 Bl/6 mice. The differences in results between the two studies may to some extent be attributed to the strain of mouse as it is well known that some strains are more efficient at killing particular microorganisms than others[281]. The results of the study by Lehrer and Jan[226], who showed that a mixture of monocytes and neutrophils was incapable of killing spores of A. fumigatus in vitro after a 3 h incubation, are in contrast to the

findings of the present study where both human PMN and monocytes were found to kill spores of A. fumigatus. However, PMN were not nearly as efficient as monocytes at killing spores of A. fumigatus, a finding which is in keeping with the results of others[223]. Schaffner et al have previously shown that mononuclear phagocytes are important in the killing of spores whilst PMN were more important in the eradication of hyphae[223]. Moreover, it is clear from clinical evidence that the PMN plays an important part in preventing invasive aspergillosis, in that the disease occurs particularly in patients with neutropenia and chronic granulomatous disease[84,86,90,91,282].

4.5 Mechanisms of Resistance

The pathogenicity of certain microorganisms has been shown to be related to their possession of antiphagocytic properties. A number of bacteria including Pseudomonas aeruginosa, Salmonella typhae, and Mycobacterium tuberculosis can inhibit chemotaxis of phagocytic cells[212,283]. Similarly Streptococcus pneumoniae, Escherichia coli and Neisseria gonorrhoeae (and many others) can attach to phagocytic cells yet resist ingestion[284]. The resistance of some microorganisms to killing is inversely related to the phagocytic cell's ability to release reactive oxygen intermediates[285,286]. Nathan and Nakagawara[287] have postulated that the pathogenicity of microorganisms, in general, may be related to their possession of antioxidant defences and to their capacity to avoid triggering the release of reactive oxygen intermediates by macrophages. It would appear from this study that A. fumigatus has evolved such mechanisms. This may be in part responsible for its pathogenicity to certain

individuals, in that it might explain why it acts as an opportunistic pathogen in immunocompromised individuals, by overcoming an already defective host defence network. It may also explain why A. fumigatus can establish itself and form a mycetoma in old disease cavities of the lung. However, it does not explain why the organism has a prediliction for asthmatic airways.

4.6 Comparisons of Fungal Handling by Asthmatic Patients

Experiments were carried out to try to establish whether the phagocytic cells of asthmatic patients sensitised to A. fumigatus are deficient at handling and, therefore, more susceptible to spores of A. fumigatus. The interaction of spores and phagocytes from asthmatic patients, both sensitised and unsensitised to A. fumigatus and from non-asthmatic subjects were compared. Phagocytes from people with asthma sensitised to A. fumigatus showed significant differences in their ability to kill A. fumigatus than did those from the control group. Monocytes from these sensitised patients killed significantly fewer spores of A. fumigatus (20%) while their PMN killed significantly more (42%). No such differences were found for P. ochrochloron. The percentage killing of fungal spores by phagocytic cells from the non-sensitised asthmatic patients generally fell between that obtained with the control and the sensitised asthmatic patient groups.

As corticosteroid treatment has been shown to depress phagocytic cell killing of A. fumigatus[153,154] the possibility that these drugs may have been contributing to the altered handling of A. fumigatus by

asthmatic patients sensitised to A. fumigatus was considered. Steroid treatment at a maintenance dose appeared to have no effect on the ability of the phagocytic cells to kill either A. fumigatus or P. ochrochloron. Earlier studies by Turner et al[155] showed that the degree of susceptibility of corticosteroid treated animals to infection by A. fumigatus could be related to the dose of corticosteroids; these workers suggested that animals on low dose corticosteroids could limit infection by A. fumigatus but treatment with high doses resulted in a high rate of mortality[155]. Similarly, patients who are on high dose corticosteroids to induce immune suppression are particularly susceptible to infection by A. fumigatus[87].

The only other reports, of which I am aware, of studies on phagocytic killing of A. fumigatus by cells from patients with aspergillus infections are on two patients with invasive aspergillosis. Both were found to have a selective defect in phagocytic killing of A. fumigatus even in the presence of specific antibody[229,230].

4.7 Role of Opsonins

From the results of the comparisons of the clinical groups it appears that PMN from patients sensitised to A. fumigatus may be compensating for the poor handling of the fungus by the monocytes. It may well have been that the presence of specific antibody in opsonising serum of these patients was augmenting this effect. Normally opsonisation of a particle with specific antibody promotes 'immune phagocytosis' via Fc receptors in the cell membrane which in turn leads to enhanced killing[174,181,209]. Plasma membrane receptors for the Fc portion of

IgG on mononuclear phagocytes have been extensively studied[288]. As well as an Fc receptor for IgG which is also found on PMN, mononuclear phagocytes possess Fc receptors for IgE[289]. Melewicz et al showed that human monocytes from allergic individuals possess Fc receptors for IgE which are involved in mediating phagocytosis and lysis of IgE coated targets[289].

Monocytes and macrophages also secrete complement components including C1q which has been found to act as a plasma membrane Fc receptor for IgG[290]. It, therefore, was unexpected given all these receptors, that monocytes from patients sensitised to A. fumigatus, showed decreased killing of A. fumigatus even in the presence of specific IgG and/or IgE antibodies. The presence of specific antibody did increase (by approximately 22%) the ability of mouse peritoneal exudate cells from naive animals to kill spores of A. fumigatus. However, specific antibody had no effect on the killing of A. fumigatus by cells from animals specifically sensitised (to A. fumigatus) as these cells showed increased killing of A. fumigatus even when opsonised in naive serum. It has previously been demonstrated that the opsonisation of spores in specific antibody does not enhance killing of A. fumigatus by phagocytic cells[227].

The study reported in this Thesis goes no further in clarifying what, if any, role specific antibody plays in the killing of A. fumigatus.

To assess further the effect of opsonins in the eradication of A. fumigatus the role of complement was investigated. It is generally considered that Fc receptors promote both binding and ingestion

of particles which have been coated with immunoglobulin, whereas complement receptors mediate attachment of complement coated particles by ingestion[291]. However, it has been shown that activated mononuclear phagocytes can ingest particles via complement receptors[292].

When spores of A. fumigatus were opsonised in serum which had been heat treated to remove complement (56°C, 30 min) the ability of phagocytic cells to kill spores of A. fumigatus was substantially increased by as much as 75%, in the case of human lung macrophages. No increased killing of similarly opsonised spores of P. ochrochloron was found. Thus instead of promoting killing the presence of heat-labile serum components actually enhanced the resistance of A. fumigatus to attack by phagocytic cells. The other serum component which is destroyed by heating at 56°C for 30 min is IgE. However, as increased killing of A. fumigatus was also found using heat-treated serum which did not contain high levels of IgE it seems unlikely that IgE was causing this effect. I have shown in unpublished experiments that spores of A. fumigatus do activate complement via the alternative pathway and also bind C3b to their surface. It, therefore, seems possible that A. fumigatus may be using complement opsonisation to its advantage in that binding via complement receptors may enable the diffusate to have more intimate contact with the cell membrane, thereby enhancing its inhibitory potential. If, however, exogenous heat labile complement components are removed the binding to phagocytic cells will be via other receptors, lectin-like receptors could be involved[293], and it may be that the position of binding within the cell membrane as well as the

degree of avidity of the spores to these other receptors does not permit the diffusate to exert such an inhibitory effect on phagocyte function. Other examples of microorganisms using complement components to their advantage exist. The protozoa Babesia rodhaini utilises C3b to gain access via C3b receptors to the erythrocyte cytoplasm where it multiplies[294]. It, therefore, seems possible that complement or other heat labile serum components, may paradoxically enhance the ability of A. fumigatus spores to survive and, therefore, germinate. In addition the results of preliminary work suggest that germination of A. fumigatus spores in culture is enhanced by the addition of serum or the products of macrophages incubated for one hour in tissue culture. Thus it appears that substances which are likely to be present in the airways of asthmatic patients may be of positive advantage to inhaled spores of A. fumigatus and enhance their germination in situ. Why this is of advantage to the organism in its natural habitat is still a matter for speculation.

4.8 Conclusions

The results of this study have shown that spores of A. fumigatus are resistant to phagocytosis and killing by both human and mouse phagocytic cells. This property seems to be due to the ability of spores to produce a low molecular weight diffusate that inhibits phagocytosis, production of reactive oxygen intermediates, phagocyte migration and macrophage spreading. It is speculated that this property of the fungus may have evolved as a mechanism of defence against its natural enemies in the soil, the amoebae. These results

may explain why A. fumigatus can overcome the already defective host defences of the immunocompromised individual and also why it is the predominant fungus associated with the development of mycetomas. It has been shown that 'paradoxically' killing of A. fumigatus by human lung macrophages is inhibited by serum which is rich in protein and enhanced if the serum is heated to temperatures that inactivate heat labile serum components including complement. This has led me to hypothesise that A. fumigatus is able not only to resist phagocytosis but can also turn the protein rich exudate within asthmatic airways to its own advantage for the purposes of survival and growth.

This work has led to the proposal of the following hypothetical sequence of events in allergic bronchopulmonary aspergillosis: first the atopic patient becomes sensitised to A. fumigatus as a result of normal day to day exposure to the organism. This then results in the development of an IgE-mediated bronchial response, with the typical asthmatic, protein-rich exudate. A. fumigatus spores not only resist phagocytosis, but also use the components of the exudate to enhance their ability to germinate and grow. The increased antigenic load thus produced intensifies the immune response and may be sufficient to provoke an IgG mediated reaction also. These responses, rather than controlling fungal growth, tend to enhance it; however, if the bronchial reactions are suppressed by corticosteroids, the proliferation of the fungus can be brought under control as the exudate subsides.

4.9 Further Studies

The sequence of events outlined above makes no mention of the role of inflammatory cells characteristic of the asthmatic exudate. The eosinophil, in particular, but also neutrophils, macrophages and mast cells play important roles in the asthmatic reaction, and their interactions with Aspergillus spores may result in more rapid elimination (or, conceivably, enhancement of germination). A further research programme has now commenced the purpose of which is to answer the following questions.

(i) What effect do cells characteristic of the inflammatory exudate of the asthmatic lung have on the germination of spores of A. fumigatus?

(ii) Do complement components enhance the ability of A. fumigatus to resist killing by phagocytic cells?

(iii) What is the biochemical nature of the inhibitory component of the spore diffusate?

It is hoped that this work will lead to a better understanding of the mechanisms of allergic bronchopulmonary aspergillosis and will aid the development of more rational methods for its management and for prevention of the serious lung damage that may ensue.

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6. APPENDIX I

Analyses of Variance Tables

Analyses of variance, for the principal results, which were obtained by the Statisticians Mr W M McLaren and Miss H P R Collins using Genstat[295] (statistical programme). These Tables were used to assess the significance of the different treatment effects in the data.

TABLE A1 Analysis of variance on the amount of chemiluminescence elicited by human monocytes and PMN in response to fungal spores, expressed as a percentage of the zymosan response

Source of variation	Sum of Square	Degrees of freedom	Mean Squares	Variance Ratio
Allergy	7.99	2	4.00	4.06
Residual (between patients)	12.79	13	0.98	
Total	20.78	15	1.39	
Cell	5.64	1	5.64	25.70
Serum	0.93	1	0.93	4.23
Spore	57.62	1	57.62	262.70
Allergy Cell	0.95	2	0.48	2.18
Allergy Serum	0.07	2	0.04	0.16
Allergy Spore	1.29	2	0.65	2.95
Cell Serum	0.02	1	0.02	0.10
Cell Spore	0.04	1	0.04	0.18
Serum Spore	0.22	1	0.22	1.01
Allergy Cell Serum	0.02	2	0.01	0.05
Allergy Cell Spore	0.11	2	0.06	0.25
Allergy Serum Spore	0.13	2	0.07	0.30
Cell Serum Spore	0.01	1	0.01	0.02
Allergy Cell Serum Spore	0.00	2	0.00	0.01
Residual (between patients)	18.86	86	0.22	
Total	85.92	107		
Grand Total	106.70	122		

TABLE A2 Analysis of variance on the results of the experiments measuring the amount of superoxide anion produced by C. parvum stimulated mouse peritoneal exudate cells in response to fungal spores

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiments (E)	2	866.8	433.4	
Treatments (T)	7	5194	742.0	75.73
E x T	11	107.8	9.798	
Residual	38	15.37	0.4045	
Total	58	6184		

TABLE A3 Analysis of variance on the results of the experiments measuring the amount of hydrogen peroxide produced by C. parvum stimulated mouse peritoneal exudate cells in response to fungal spores

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiments (E)	2	367.44	183.72	
Treatments (T)	6	414.63	69.10	4.86
E x T	9	128.27	14.25	
Residual	35	11.33	0.324	
Total	52	921.67		

TABLE A4 Analysis of variance on the superoxide anion produced by monocytes and PMN, in response to zymosan spores of A. fumigatus and P. ochrochloron, from patients in the three clinical groups including effect of serum treatment.

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Subject Stratum				
GRP1	3	0.7379	0.2460	0.274
Residual	22	19.7574	0.8981	
Total	25	20.4953	0.8198	
Subjects *Units *Stratum				
Treatment	5	27.4199	5.4840	51.631
Cell (Monocyte PMN)	1	19.0743	19.0743	179.581
GRP1 Treatment	15	0.4673	0.0312	0.293
GRP1 Cell	3	1.3500	0.4500	4.237
Treatment Cell	5	5.4776	1.0955	10.314
GRP1 Treatment Cell	15	0.6300	0.0420	0.395
Residual	221 (21)	23.4737	0.1062	
Total	265	77.8928	0.2939	
Grand Total	290	98.3881		

TABLE A5 Analysis of variance of the results of the experiments measuring the effect of spore diffusates on the production of superoxide anion by C. parvum stimulated mouse peritoneal exudate cells

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiment (E)	1	10.778	10.77	
Treatment (T)	2	232.93	116.46	126.19
E x T	2	1.85	0.92	
Residual	11	3.018	0.274	
Total	16	248.57		

TABLE A6 Analysis of variance of the results of the experiments measuring the effect of spore diffusates on the production of hydrogen peroxide by C. parvus stimulated mouse peritoneal exudate cells

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiments (E)	4	1157	289.1	
Treatments (T)	2	2207	1103	19.77
E x T	8	446.4	55.8	
Residual	29	20.5	0.7068	
Total	43	3830		

TABLE A7 Analysis of variance of the chemotaxis of human PMN towards zymosan activated serum : effect of spore diffusates

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiments	2	671.24	335.62	13.63
Treatments (adjusted for experiments)	2	9794.02	4897.01	198.90
Error	36	886.28	24.62	
Total	40	11351.53		

TABLE A8 Analysis of variance of the spreading of C. parvum stimulated mouse peritoneal exudate cells on glass : effect of spore diffusates

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Experiment	2	32.3	16.1	15.6
Treatments (adjusted for experiments)	2	425.4	212.7	205.3
Error	12	12.4	1.0	
Total	16	470.2		

TABLE A9 Analysis of variance of killing of spores of A. fumigatus and P. ochrochloron by human pulmonary macrophages including effect of serum treatment

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Patient Stratum				
Sex	1	71.11	71.11	0.028
Residual	8	20616.55	2577.07	
Total	9	20687.66	2298.63	
Patient *Units* Stratum				
Spore	1	12965.40	12965.40	152.853
Heat	1	0.00	0.00	0.000
Sex.spore	1	1307.21	1307.21	15.411
Sex.heat	1	0.00	0.00	0.000
Spore.heat	1	0.00	0.00	0.000
Sex.spore.heat	1	0.00	0.00	0.000
Sex.spore.temp	1	0.00	0.00	0.000
Spore.heat.temp	1	0.00	0.00	0.000
Residual	41	3477.72	84.82	
Total	50	17750.33	355.01	
Grand Total	59	38438.00		

TABLE A10 Analysis of variance on the killing of spores of A. fumigatus and P. ochrochloron by monocytes and PMN from patients divided by clinical group including effect of serum treatment

Source of variation	Degrees of freedom	Sum of Square	Mean Squares	Variance Ratio
Subject stratum				
GRPNew	3	7888.9	2629.6	0.314
Residual	38	318353.4	8377.7	
Total	41	326242.3	7957.1	
Subject *Units* Stratum				
Spore	1	3618.6	3618.6	12.282
Cell	1	0.0	0.0	0.000
Serum	1	0.0	0.0	0.000
GRPNew.Spore	3	3195.6	1065.2	3.615
GRPNew.Cell	3	10.2	3.4	0.012
Spore.Cell	1	25.7	25.7	0.087
GRPNew.Serum	3	25.2	8.4	0.028
Spore.Serum	1	337.1	337.1	1.144
Cell.Serum	1	0.0	0.0	0.000
GRPNew.Spore.Cell	3	52.6	17.5	0.060
GRPNew.Spore.Serum	3	74.8	24.9	0.085
GRPNew.Cell.Serum	3	0.0	0.0	0.000
Spore.Cell.Serum	1	0.0	0.0	0.000
GRPNew.Spore.Cell.Serum	3	5.8	1.9	0.007
Residual	218 (48)	64230.2	294.6	
Total	246	71575.9		
Grand Total	287	397818.1		

7. APPENDIX II

Scientific publications from this work

(i) ROBERTSON M D, RAEBURN J A, GORMLEY I P G, SEATON A. Do phagocytic cells ingest spores of A. fumigatus? (abstract). Thorax 1985; 40: 237.

(ii) ROBERTSON M D, SEATON A, MILNE L J R, RAEBURN J A. Suppression of host defences by A. fumigatus. Thorax 1987; 42: 19-25.

(iii) ROBERTSON M D, KERR K M, SEATON A. The effect of complement on the killing of Aspergillus (abstract). Thorax 1987; 42: 213.

(iv) ROBERTSON M D, SEATON A, MILNE L J R, RAEBURN J A. Resistance of spores of Aspergillus fumigatus to ingestion by phagocytic cells. Thorax 1987; 42: 466-472.

(v) ROBERTSON M D, SEATON A, RAEBURN J A, MILNE L J R. Inhibition of phagocyte migration and spreading by spore diffusates of Aspergillus fumigatus. J Med Vet Mycol 1987. In press

Thorax 1987;**42**:19-25

Original articles

Suppression of host defences by *Aspergillus fumigatus*

MAURA D ROBERTSON, A SEATON, L J R MILNE, J A RAEBURN

From the Institute of Occupational Medicine and the Western General Hospital, Edinburgh

ABSTRACT An important feature of the microbicidal action of phagocytic cells is their ability to produce reactive oxygen intermediates. In an attempt to identify the mechanisms by which the fungus *Aspergillus fumigatus* resists normal host defences the effect of spores and spore diffusates of *A fumigatus* on the production of superoxide anion and hydrogen peroxide by primed rodent phagocytic cells has been measured. For comparison we have used the non-pathogenic fungus *Penicillium ochrochloron*. Production of these reactive oxygen intermediates in response to *A fumigatus* was significantly lower than that in response to *P ochrochloron*. A similar reduction was achieved by diffusate prepared from freshly washed spores. The inhibitory component was of low molecular weight (less than 14000) and its effect was dose dependent. These results suggest that spores of *A fumigatus* fail to trigger and also inhibit the production of reactive oxygen intermediates by phagocytic cells.

The opportunistic fungus *Aspergillus fumigatus* is of special interest to respiratory physicians because of its ability to cause several distinct pulmonary diseases. It may cause sensitisation in asthmatic subjects, leading in some instances to chronic symptoms and recurrent episodes of pulmonary infiltration.[1] It may also colonise lung cavities to produce aspergillomas[2] and invade lung tissue after infarction[3] or influenza[4] to produce a fatal necrotising pneumonia, or cause pulmonary and systemic infection in the immunosuppressed.[5] The frequency with which it may be associated with lung disease suggests that the organism may be relatively resistant to the lung's defences. While spores of *A fumigatus* occur commonly in the air, especially in winter, when dead leaves are available as a substrate, they are not among the most abundant.[6] Moreover, studies of postmortem lung specimens have shown *A fumigatus* to be present and viable more frequently than would be expected from its prevalence among the fungal spores normally found in the air, the aerospora, thus supporting the idea that it may have properties that protect it from the lung's defences.[7] Such properties could ensure its survival in the lung long enough to initiate allergic and infective reactions in appropriate hosts.

Two factors that enhance the pathogenicity of *A fumigatus* in man are the organisms' ability to grow optimally at 37°C and the small spore size that enables it to penetrate to the alveolar level. To survive and germinate in the lung the spores must also be resistant to the phagocytic defences of the lung. In this paper we report the results of in vitro investigations of the interactions between *A fumigatus* and phagocytic cells from rodents.

Address for reprint requests: Mrs MD Robertson, Institute of Occupational Medicine, 8 Roxburgh Place, Edinburgh EH8 9SU.

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Methods

GENERAL PLAN

In an attempt to identify the mechanisms used by this fungus to resist killing by phagocytic cells we have examined the effects of spores and spore diffusate of *A fumigatus* on the ability of the cell to produce oxidising agents (reactive oxygen intermediates) that can be used by the phagocyte for the destruction of microorganisms.[8] We have compared the release by phagocytic cells from mice and rats of the reactive oxygen intermediates superoxide anion and hydrogen peroxide in response to challenge by *A fumigatus* and by the non-pathogenic fungus *Penicillium ochrochloron*.

PHAGOCYTIC CELLS

Since resting phagocytic cells release minimal amounts of reactive oxygen intermediates, we used phagocytic cells obtained from mice and rats that had been previously primed in vivo with *Corynebacterium parvum*. This is a procedure that has been shown to increase the ability of phagocytic cells to release reactive oxygen intermediates,[9] as well as generally to

increase their microbicidal capacity.[10 11] For each experiment about 4×10^7 peritoneal exudate cells, which comprise 32% polymorphonuclear leucocytes and 63% macrophages, were harvested from six male C57Bl/6 mice five days after intraperitoneal injection of 1·4 mg *C parvum* (heat killed, Wellcome Biotechnology Ltd). About 2×10^7 bronchoalveolar lavage cells (90% of which were polymorphonuclear leucocytes) were obtained from the lungs of male PVG rats 16 hours after intratracheal instillation of 0·7 mg *C parvum*.

FUNGAL SPORES AND DIFFUSATES

A single strain of *A fumigatus* was isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis. The strain of *P ochrochloron* was IMI 61271. Spores of *A fumigatus* and *P ochrochloron* were obtained from cultures that had been grown for seven days at 29°C on malt agar. Spore suspensions containing only single spores were prepared in Hanks' balanced salt solution (HBSS) containing 0·01% bovine serum albumin (Sigma Chemical Co Ltd), after gentle homogenisation followed by filtration through nylon mesh to remove mycelial fragments and chains of spores. Spore counts were performed with improved Neubauer chambers. For the spore:cell experiments the spores were opsonised in 5% autologous serum for 30 minutes at 37°C, then washed in HBSS by centrifugation at 400 *g* for 10 minutes. The spores were then resuspended in HBSS and kept on ice until required. To obtain diffusates, spores were incubated at a concentration of 10^8/ml in HBSS at 37°C for up to six hours. The soluble diffusates in the supernatant were passed through 0·22 μm sterile filters. For the assay systems the diffusates were used at a 1:4 dilution in HBSS. The number of spores require to obtain this concentration of diffusate was equivalent to the number derived from a spore:cell ratio of 50:1.

SUPEROXIDE ANION ASSAY

Superoxide anion was measured according to the method of Johnston.[12] Production of superoxide anion can be detected by the reduction of cytochrome C, a reaction accompanied by an increase in spectrophotometric absorbance at a wave length of 550 nm. The reaction mixture (1·5 ml), containing 5×10^5 peritoneal exudate cells, 80 μmol cytochrome C (Sigma), 2 mg/ml dextrose, and opsonised spores at a spore:cell ratio of 10:1, 50:1, and 100:1 or spore diffusates at a 1:4 dilution, was added to 30 mm Petri dishes (Falcon Plastics Ltd). For the diffusate dose-effect experiments a total reaction mixture of 1·3 ml containing $2·5 \times 10^5$ bronchoalveolar cells was used. As an additional control, zymosan (Sigma), a cell wall extract of the yeast *Saccharomyces cerevisiae* and a potent trigger of superoxide anion release, was opsonised and added to the reaction mixture at a concentration of 1 mg ml. To determine the portion of reduced cytochrome C that could be inhibited by superoxide dismutase, identical dishes with the addition of superoxide dismutase (Sigma) 25 μg ml were prepared at the same time. All experiments were set up in triplicate and the mixtures incubated at 37 C for two hours. The supernatants were harvested and the peak absorbance at 550 nm, a measure of reduced cytochrome C, was determined by using the scan mode on a spectrophotometer (SP8 400 Pye Unicam). The reduction of cytochrome C is not specific for superoxide anion. The required specificity is achieved by the use of superoxide dismutase, for which superoxide anion is the only known substrate. Therefore the assay was run with and without superoxide dismutase and only the portion of the reduced cytochrome C that could be inhibited by superoxide dismutase was used to estimate the nanomols of superoxide anion released.

HYDROGEN PEROXIDE ASSAY

Hydrogen peroxide was measured according to the method of Pick and Keisari.[13] The assay is based on the horseradish peroxidase mediated oxidation of phenol red by hydrogen peroxide, which results in the formation of a compound showing increased absorbance at 610 nm. The reaction mixture (1 ml), containing 5×10^5 peritoneal exudate cells, 10 nmol phenol red solution (Sigma), NaCl (140 nmol), dextrose (5·5 nmol), and horseradish peroxidase (50 μg ml) (Sigma) in 10 nmol potassium phosphate buffer, and opsonised spores at a spore:cell ratio of 10:1, 25:1, and 50:1 or spore diffusates at a 1:4 dilution was added to 30 mm Petri dishes. For the diffusate dose-effect experiments a total reaction mixture of 1·3 ml containing $2·5 \times 10^5$ bronchoalveolar cells was used. As an additional control, opsonised zymosan was added to the reaction mixture at a concentration of 1 mg ml. All experiments were set up in triplicate and incubated at 37°C for two hours. The supernatants were then harvested and alkalised by the addition of 30 μl of 1N NaOH. The absorbance of the solution at 610 nm was measured in a spectrophotometer and converted to hydrogen peroxide equivalent by means of a standard curve.

TECHNIQUES USED TO DETECT A POSSIBLE SCAVENGING EFFECT OF DIFFUSATES ON REACTIVE OXYGEN INTERMEDIATES

To test for a possible scavenging effect of the diffusates on reactive oxygen intermediates, we used: (*a*) a cell free superoxide anion generation system generally based on the method described by Rosen and Klebanoff.[14] Superoxide anion was generated by incubating xanthine oxidase 20 μg/ml (Sigma) with

acetaldehyde 40 nmol for five minutes at 37°C. This resulted in the generation of about 6 nmol. To this superoxide anion 1 ml of the following was added: (*a*) HBSS (control); (*b*) superoxide dismutase (25 μg), which catalyses the destruction of superoxide anion to oxygen and hydrogen peroxide; (*c*) diffusate (1:4 dilution). Each of the three treatments was set up in triplicate and incubated for 10 minutes at 37°C. The reaction mixture, used to detect superoxide anion (described above), was then added and after a further incubation for 10 minutes at 37°C the absorbance at 550 nm was measured; (ii) hydrogen peroxide (Sigma) was diluted to give concentrations ranging from 1 to 20 nmol. To the hydrogen peroxide 1 ml of the following was added: (*a*) HBSS (control); (*b*) catalase (Sigma) 100 μg/ml, an enzyme that catalyses the degradation of hydrogen peroxide to oxygen and water; and (*c*) diffusate (1:4 dilution). Each of the three treatments was prepared in duplicate and incubated for 30 minutes at 37°C. The reaction mixture used to detect hydrogen peroxide (described above) was added to the tubes, which were then incubated for a further 30 minutes at 37°C. The absorbance of the mixture was read at 610 nm.

DIFFUSATE DIALYSIS

To determine whether the active constituent of the diffusate was of low molecular weight the diffusate was dialysed against HBSS for 16 hours at 4°C. As we had observed that the activity of the diffusate decreases on storage at 4°C, an aliquot of the diffusate was put into dialysis tubing and kept in a sealed tube for 16 hours at 4°C, as a control.

DIFFUSATE RELEASE

To discover at what stage the diffusates were released into the supernatant we added spores (10^8/ml) to HBSS, divided this suspension into three aliquots, and carried out the following treatments. (1) The spores were incubated for two minutes and immediately removed and the supernatants filtered ("two minutes incubation (washings)" in table 4). (2). The washed spores were resuspended in HBSS and incubated for three hours at 37°C before the spores were spun out to obtain the supernatant, which was filtered ("washed + three hours incubation"). (3) The spores were incubated in HBSS for 3 hours and the supernatant removed then filtered ("three hours incubation").

CELL VIABILITY

To determine whether the spore diffusates were having a toxic effect on the cells, 5×10^5 cells were incubated for two hours at 37°C in 1 ml of control HBSS or spore diffusates (1:4 dilution). After this time the percentage viability was determined by the trypan blue (Gibco) exclusion method.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Each experiment was carried out in triplicate and performed on two to five separate occasions. For individual experiments a different group of animals and spores or spore diffusates prepared from fresh cultures were used. Therefore day to day variation in spontaneous release of reactive oxygen intermediates and strength of diffusates did occur. The statistical analyses were designed to take into account this variation between experiments and treatments so that the analyses of treatment effects would be independent of inter-experimental variation. Initially the raw data were subjected to analysis of variance with a randomised block design in which each day/experiment constituted a block. Student's *t* tests were used to compare treatment means, using the residual error obtained from the analysis of variance.

Results

EFFECTS OF SPORES ON RELEASE OF SUPEROXIDE ANION

C parvum stimulated mouse peritoneal exudate cells released significantly less superoxide anion ($p < 0{\cdot}001$) in response to spores of *A fumigatus* than in response to zymosan or *P ochrochloron* (table 1). Both zymosan and *P ochrochloron* were associated with a release of superoxide anion that was significantly greater than the spontaneous release by cells ($p < 0{\cdot}001$). When the results from the individual spore:cell ratios were combined, *P ochrochloron* was seen to stimulate an increase in superoxide anion release of about 50%, while zymosan, a potent trigger of superoxide anion, stimulated a three fold increase.

EFFECTS OF SPORES ON RELEASE OF HYDROGEN PEROXIDE

Both zymosan and *P ochrochloron* slightly reduced the spontaneous release of hydrogen peroxide by *C par-*

Table 1 *Effect of opsonised zymosan and opsonised fungal spores on the release of superoxide anion by peritoneal exudate cells*

Treatment	*Spore : cell ratio*	*Superoxide anion (nmol : mean (SEM))*
HBSS*	—	12·89 (1·20)
Zymosan	—	41·6 (0·60)
A fumigatus	10 : 1	12·06 (0·20)
A fumigatus	50 : 1	11·07 (0·45)
A fumigatus	100 : 1	11·09 (0·84)
P ochrochloron	10 : 1	14·62 (1·60)
P ochrochloron	50 : 1	20·12 (1·23)
P ochrochloron	100 : 1	18·4 (1·70)

*Treatment with Hanks' balanced salt solution (HBSS) gives a measure of the spontaneous release.

Table 2 *Effect of opsonised zymosan and opsonised fungal spores on the release of hydrogen peroxide by peritoneal exudate cells*

Treatment	Spore: cell ratio	Hydrogen peroxide (nmol: mean (SEM))
HBSS*	—	10·19(1·57)
Zymosan	—	9·53(1·53)
A fumigatus	10:1	8·63(1·41)
A fumigatus	25:1	6·80(0·35)
A fumigatus	50:1	5·15(0·87)
P ochrochloron	10:1	8·63(1·18)
P ochrochloron	50:1	8·16(1·88)

*Treatment with Hanks' balanced salt solution (HBSS) gives a measure of the spontaneous release.

vum stimulated mouse peritoneal exudate cells (table 2). *A fumigatus* produced a reduction of hydrogen peroxide release and this effect was more pronounced with increasing spore:cell ratios. When the results from the individual spore:cell ratios were combined the release of hydrogen proxide was significantly lower in response to *A fumigatus* ($p < 0·001$) than to zymosan or *P ochrochloron*.

EFFECTS OF SPORE DIFFUSATES ON RELEASE OF REACTIVE OXYGEN INTERMEDIATES

To examine the possibility that *A fumigatus* may produce a substance that has a direct effect on cellular production of reactive oxygen intermediates, we measured the effect of diffusates obtained from spores of *A fumigatus* and *P ochrochloron* on the spontaneous release of reactive oxygen intermediates from *C parvum* stimulated mouse peritoneal exudate cells. Diffusates from *A fumigatus* greatly reduced ($p < 0·001$) the spontaneous release of superoxide anion and hydrogen peroxide (table 3) while those from *P ochrochloron* did not.

Using bronchalveolar lavage cells obtained from rats whose lungs had been treated with *C parvum*, we found that this inhibitory effect was strikingly dependent on the concentration of diffusate (fig 1).

STUDIES OF THE DIFFUSATE FROM SPORES OF A FUMIGATUS

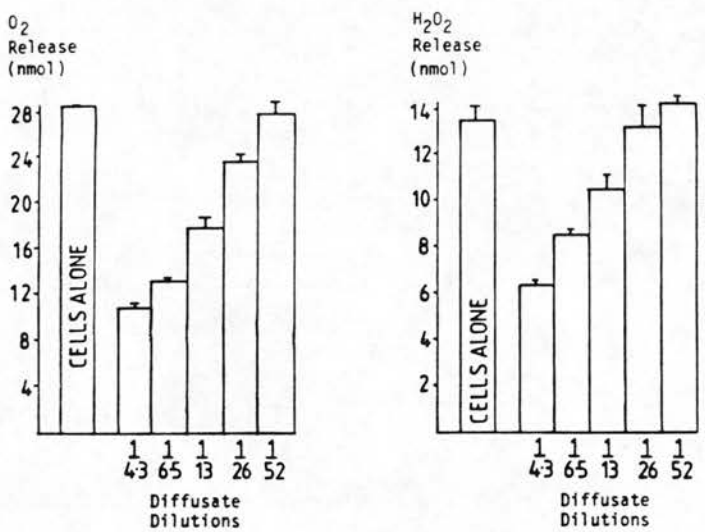
(1) Viability studies established that the spore diffusates were not having a toxic effect on the cells, as the percentage viability of the cells after an incubation period of two hours in spore diffusate was the same as that found with control HBSS (>95% viable).

(2) To find out at what stage the diffusates were released into the supernatant, we looked at the effect of time on the diffusion, from spores of the factor that inhibited reactive oxygen intermediates production.

Table 3 *Effect of spore diffusates on the spontaneous release of superoxide anion and hydrogen peroxide by peritoneal exudate cells*

Treatment	Superoxide anion (nmol: mean (SEM))	Hydrogen peroxide (nmol: mean (SEM))
HBSS*	14·38(0·26)	18·82(1·63)
A fumigatus diffusates	6·73(0·43)	4·16(0·52)
P ochrochloron diffusates	14·40(0·67)	19·43(2·25)

*Treatment with Hanks' balanced salt solution (HBSS) gives a measure of the spontaneous release.



Effect of increasing dilution of spore diffusates of Aspergillus fumigatus on the spontaneous release of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) by phorbol myristate acetate triggered Corynebacterium parvum induced rat bronchoalveolar lavage cells ($2·5 \times 10^5$ cells) when compared with the cells alone (two hour incubation period): means with standard deviations.

Table 4 shows that washings (2 min incubation) from the spores significantly reduced release of reactive oxygen intermediates ($p < 0.001$) by *C parvum* stimulated mouse peritoneal exudate cells. Therefore the diffusate is released immediately the spores are put into liquid. In addition, the spores, once washed, continue to release the diffusate. The longer the spores were incubated the greater was the inhibitory capacity of the diffusate.

(3) To examine whether the diffusate was affecting production of reactive oxygen intermediates by the phagocytic cell or scavenging the reactive oxygen intermediates once produced, or both, we looked at the effect of adding the diffusate at a 1:4 dilution to a cell free superoxide anion generation system and to hydrogen peroxide. We found that the amount of superoxide anion present after the addition of the diffusates (5·84 nmol) was the same as that found after the addition of control HBSS (5·86 nmol). The addition of superoxide dismutase (which is used in the conversion of superoxide anion to hydrogen peroxide), however, removed the superoxide anion from the system. This result confirmed that the diffusate does not scavenge superoxide anion as generated by the xanthine oxidase acetaldehyde system. Similarly, we found that the diffusates did not scavenge hydrogen peroxide. The addition of control HBSS to a system containing hydrogen peroxide results in a linear increase in absorbance at 610 nm (a measure of hydrogen peroxide in the system) with increasing concentration of hydrogen peroxide (1–20 nmol). The addition of the diffusate to the hydrogen peroxide resulted in an increase in absorbance at 610 nm, the same as that given with HBSS. When catalase, an enzyme that breaks down hydrogen peroxide, was added to the system, however, no increase in absorbance at 610 nm was obtained.

(4) The inhibitory effect of the diffusate on release of reactive oxygen intermediates by *C parvum* stimulated mouse peritoneal exudate cells was removed by dialysis (table 5), indicating that the molecular weight of the active substance in the diffusate is less than 14 000 daltons.

Table 4 *Effect of time on the diffusion from A fumigatus spores of activity that inhibits the production of superoxide anion and hydrogen peroxide by peritoneal exudate cells*

Treatment	*Superoxide anion (nmol mean SEM)*	*Hydrogen peroxide (nmol mean SEM)*
HBSS*	13·43 (0·42)	13·9 (0·69)
2 min incubation (washings)	6·93 (0·25)	8·6 (0·36)
washed spores + 3 h incubation	5·13 (0·18)	4·43 (0·14)
3 h incubation	4·77 (0·15)	3·3 (0·06)

*Treatment with Hanks' balanced salt solution (HBSS) gives a measure of the spontaneous release

Table 5 *Effect of dialysis on the capacity of the diffusate to inhibit the production of superoxide anion and hydrogen peroxide*

Treatment	*Superoxide anion (nmol mean SEM)*	*Hydrogen peroxide (nmol mean SEM)*
HBSS*	12·98 (1·38)	21·3 (2·06)
Diffusates	5·65 (0·57)	8·75 (1·07)
Diffusates incubated 4 C 18 h	9·95 (1·28)	13·86 (0·38)
Diffusates dialysed 4 C 18 h	13·76 (1·69)†	25·6 (1·82)†

*Treatment with Hanks' balanced salt solution (HBSS) gives a measure of the spontaneous release
†$p < 0.0001$ for incubated versus dialysed diffusate

Discussion

The key role played by phagocytic cells in the eradication of microorganisms is well documented.[8 15–17] Several studies have, however, reported apparently conflicting results concerning the interaction of these cells with *A fumigatus*. Rabbit alveolar macrophages[18] and human blood monocytes[19] have been found to be capable of killing *A fumigatus* in vitro. Schaffner *et al*,[20] who compared natural immunity to *A fumigatus* in vivo with the actions of phagocytes against the organisms in vitro, suggested that monocytes may be concerned with killing spores while polymorphonuclear leucocytes may be responsible for the eradication of hyphae, apparently in the absence of a specific immune response. Other workers, however, have been unable to show either in vitro killing of *A fumigatus* by human monocytes or polymorphonuclear leucocytes[21] or killing in vivo by mouse or rabbit alveolar cells.[22 23] Nevertheless, the potential importance of reactive oxygen intermediates, and of hydrogen peroxide in particular, in the eradication of spores and hyphae has been demonstrated with cell free systems of myeloperoxidase-hydrogen peroxide-halide[24] and ferrous iron-hydrogen peroxide-halide,[25] both of which are capable of killing *A fumigatus*.

Our study, using phagocytic cells from mice and rats, shows that spores of *A fumigatus* fail to trigger an increase in superoxide anion release while zymosan and control spores of *P ochrochloron* trigger a substantial increase. This suggests that spores of *A fumigatus* are failing to trigger the primary component of the "respiratory burst." The normal sequence of events in the release of reactive oxygen intermediates by phagocytic cells is the production of superoxide anion, which can either spontaneously or with the help of superoxide dismutase form hydrogen peroxide.[16] If, as we have found, *A fumigatus* does not trigger an increased release of detectable amounts of

superoxide anion it might be expected that hydrogen peroxide concentrations would also be low, unless rapid dismutation of superoxide anion were occurring. We have shown that spores of *A fumigatus*, as well as failing to trigger superoxide anion release, also at a spore:cell ratio of 25:1 and above suppress hydrogen peroxide production by comparison with either spores of *P ochrochloron* or zymosan. The responses of stimulated peritoneal exudate cells to triggering by zymosan with the release of increased amounts of superoxide anion without an increase in the release of hydrogen peroxide have been previously reported both from this laboratory[9] and by others.[26] In general, the release of reactive oxygen intermediates in response to spores of the control fungus *P ochrochloron* parallels the response to zymosan.

We have previously noted that spores of *A fumigatus* become attached to phagocytic cells[27] in vitro and in vivo; it is therefore interesting that binding of spores to the cell does not trigger release of reactive oxygen intermediates. The studies reported here explain this to the extent that we have shown that the spores release a substance that interferes with the production of those oxidants, and that this substance is released immediately the spores are put into suspension. Furthermore, it continues to be released after the spores have been washed and is of low (less than 14 000 daltons) molecular weight. Thus the physical interactions of spores of *A fumigatus* with the phagocytic cells appear to produce an immediate respiratory burst similar to that produced by cells alone. The enhanced production of superoxide anion seen in respose to the controls *P ochrochloron* and zymosan is, however, suppressed in response to release of the diffusate by *A fumigatus*. These findings are consistent with our previous observations that, in vitro, a large number of spores of *A fumigatus* appear to remain bound to the surface of phagocytic cells without becoming fully ingested.[27] This potential antiphagocytic effect of *A fumigatus* has also recently been described by Müllbacher *et al*.[28] who found that a metabolite called gliotoxin, isolated from three day culture supernatants of *A fumigatus*, had an inhibitory effect on the phagocytosis of carbon particles by mouse peritoneal exudate cells. The difference between this gliotoxin and the diffusate described in the present study is that the gliotoxin could not be isolated until the spores had been in culture for at least three days, at which time mycelial growth would be abundant. In contrast, the diffusate described here diffuses from the respirable sized spores as soon as they are put into suspension. The quick release of the diffusate from the spores suggests that it may be located on or close to the surface of the spore. Possibly the diffusate is the first mechanism by which the spore reduces the efficiency of the phagocyte while the gliotoxin, which is produced once germination has taken place, may be a second line of antiphagocyte defence used by *A fumigatus* to establish itself and remain within the lung.

Our findings may also be relevant to the results of those studies that have indicated that the resistance of some microorganisms to killing is inversely related to the phagocytic cell's ability to release reactive oxygen intermediates.[29,30] Nathan and Nakagawara[17] have postulated that the pathogenicity of microorganisms may, in general, be related to their possession of antioxidant defence and to their capacity to avoid triggering the release of reactive oxygen intermediates by macrophages. *A fumigatus* would appear from our study to have evolved such mechanisms and this may in part be responsible for its pathogenicity to the lungs of certain individuals. Probably normal individuals have sufficient resources within their host defence network to overcome and remove these spores. At the other end of the scale *A fumigatus* can overcome the defective host defences of immunocompromised individuals, resulting in invasive aspergillosis. Perhaps some patients with asthma lie in the middle of this hypothetical scale, where the fungus, although controlled to a certain degree, is not efficiently removed and therefore remains within the lung, thus enabling it to provoke an immune response with the resultant allergic reactions.

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Resistance of spores of *Aspergillus fumigatus* to ingestion by phagocytic cells

MAURA D ROBERTSON, ANTHONY SEATON, L J R MILNE, J A RAEBURN

From the Institute of Occupational Medicine and the Western General Hospital, Edinburgh

ABSTRACT Phagocytic cells are believed to have an important role in the eradication of fungal spores from the lung. The ability of human and mouse cells to phagocytose the opportunistic fungus *Aspergillus fumigatus* has been examined, spores of the non-pathogenic fungus *Penicillium ochrochloron* being used for comparison. Most spores became associated with cells. Those of *A fumigatus* appeared to remain bound to the surface of the phagocyte rather than being ingested; in contrast, *P ochrochloron* spores appeared to be phagocytosed more readily, although they also were seen, in smaller numbers, on the cell surface. In view of the subjective nature of these observations, the effects of spore diffusates on phagocytosis were examined. Diffusates from spores of *A fumigatus* were shown to inhibit phagocytosis of antibody coated radiolabelled sheep red blood cells by primed mouse phagocytic cells. Diffusates of spores of *P ochrochloron* had no such effect. These results suggest that when spores of *A fumigatus* become bound to the surface of phagocytes they are able to release a substance that inhibits their ingestion while having little or no effect on surface binding.

Inhalation of spores of the opportunistic fungus *Aspergillus fumigatus* may lead to allergic disease in man[1] and in the immunosuppressed may cause life threatening infection.[2] While phagocytic cells are clearly important in host defences against invading microorganisms,[3-5] their precise role in the killing of *A fumigatus* remains undefined.[6-11] Phagocytosis is often an important step in the killing of microorganisms, and this has generally been assumed to be the case with inhaled fungal spores. In one study, however, no evidence of phagosome-lysosome fusion was found in relation to phagocytosis of *A fumigatus*,[12] and recent studies from our laboratories have shown that this organism is able to produce a diffusate that significantly inhibits the respiratory burst of phagocytic cells.[13] *A fumigatus* spores moreover, as we have suggested in a preliminary report, may be particularly resistant to phagocytosis.[14]

The process of phagocytosis can be divided into two phases: (1) attachment of the particle to the cell surface, followed by (2) ingestion. Steps 1 and 2 are collectively referred to as cell association. Only when a particle has undergone step 2 can it be said to be phagocytosed. Nevertheless, the term phagocytosis is often used synonymously with cell association, implying that the particle is phagocytosed when the techniques used are unable to distinguish clearly between attached and ingested particles. In this paper we report further studies of the interactions between spores of *A fumigatus* and phagocytic cells, with particular reference to the two phases of the phagocytic process.

Address for reprint requests: Mrs M D Robertson, Institute of Occupational Medicine, 8 Roxburgh Place, Edinburgh EH8 9SU.

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Methods

The study was designed to assess, by light and electron microscopy, the ability of phagocytic cells to ingest spores of *A fumigatus* and, for comparison, *P ochrochloron*. Since this assessment is necessarily subjective, a well established method of assessing the number of ingested labelled sheep red blood cells accurately has been used to investigate the effects of diffusates of these spores on the phagocytic process.

PHAGOCYTIC CELLS AND SERA

Human monocytes and polymorphonuclear leucocytes were isolated from the peripheral blood of six healthy donors by density gradient centrifugation.[15] Autologous serum was obtained from a clotted blood sample at the same time. Serum containing specific antibody to *A fumigatus* was obtained from a patient

with an aspergilloma, and this produced about 10 precipitin lines when tested against a standard *A fumigatus* antigen preparation (Bencard Allergy Division).

Peritoneal exudate cells were harvested from 12 week old syngeneic C57B1/6 mice by lavaging the peritoneal cavity with isotonic saline. The mice were pretreated by intraperitoneal injection of either 3% thioglycollate broth (0·5 ml, Difco Products) four days before harvesting or *Corynebacterium parvum*, 0·2 ml of 7 mg/ml (Wellcome Biotechnology Limited) five days before harvesting.

FUNGAL SPORES AND DIFFUSATES

The method used was that described previously,[13] with the slight modification that after opsonisation spores of *A fumigatus* and *P ochrochloron* were used directly in the assays, suspended in RPMI-1640–5% serum.

ESTIMATION OF CELL ASSOCIATION OF FUNGAL SPORES IN VITRO

Fifty microlitres of human phagocytic cells (1×10^7/ml in RPMI–5% autologous serum) were added to microtitre plates. At the same time control wells containing 50 μl of RPMI–5% serum alone were set up. To all wells 100 μl of opsonised spores (5×10^6/ml in RPMI–5% serum) were added to give a final spore:phagocytic cell ratio of 2:1 or 1:1. To accelerate the contact process between spores and cells microtitre plates were centrifuged at 125 *g* for five minutes, then incubated at 37°C for one hour. After gentle mixing to resuspend sedimented spores, an aliquot of supernatant was removed and the number of free spores present was counted with an improved Neubauer counting chamber. Total spores were estimated from the number of spores in the supernatant of the control wells containing spores alone; this was used as a baseline to determine the percentage of spores becoming cell associated, the following formula being used:

$$\% \text{ of spores cell associated} = \left(\frac{\text{total spores} - \text{spores non-cell associated}}{\text{total spores}} \right) \times 100.$$

All experiments were carried out in triplicate.

MICROSCOPY OF CELL-SPORE PREPARATIONS

Phagocytic cells (200 μl of 5×10^6/ml in RPMI–5% autologous serum) were allowed to adhere to glass microchamber slides (LAB-TEK) or glass coverslips (13 mm diameter) for one hour at 37°C in 5% carbon dioxide. The coverslips were then gently rinsed twice in warm Hanks' balanced salt solution (HBSS) to remove non-adherent cells. Opsonised spores in RPMI–5% autologous serum were added to the adherent phagocytic cells at a spore:cell ratio of 2:1–1:1 and the cultures incubated at 37°C in 5% carbon dioxide for 1·5 hours. After this period excess spores were removed by gently washing the cultures in warm HBSS three times. The slides were fixed in methanol and stained in May-Grünwald-Giemsa.[16] The coverslips were processed for scanning electron microscopy[17] by fixing at 37 C for 30 minutes in 2·5% glutaraldehyde (Sigma) in 0·1 mol l cacodylate buffer containing 0·1 mol l sucrose (Sigma) followed by two rinses in the buffer. The coverslips were then dehydrated in graded series of acetone-water mixtures and critically point dried with liquid carbon dioxide. This method was used because light microscopy does not differentiate between spores that have been phagocytosed and those that remain on the surface of phagocytes.

PHAGOCYTOSIS OF FUNGAL SPORES IN VIVO

C57B1/6 mice that had received an intraperitoneal injection of 3% thioglycollate (0·5 ml) four days previously were challenged intraperitoneally with 10^8 spores in 0·5 ml HBSS. After 1·5 hours the peritoneal cavities were lavaged with 5 ml of HBSS; the lavaged cell population with attached or ingested spores was allowed to adhere to coverslips for one hour at 37 C and was then prepared for light and scanning electron microscopy.

PHAGOCYTOSIS OF RADIOLABELLED ANTIBODY COATED SHEEP RED BLOOD CELLS

The method was based on that described by Shaw and Griffin.[18] Sheep red blood cells in Alsever's solution (Tissue Culture Service) were washed three times in phosphate buffered saline (PBS). The washed packed sheep red blood cells were radiolabelled by incubation in $^{51}Cr\ Na_2\ CrO_4$ (10^8 sheep red blood cells 100 μCi ^{51}Cr) (Amersham International) for one hour at 37°C. The ^{51}Cr labelled sheep red blood cells were washed once in PBS and adjusted to 10^9 ^{51}CR labelled sheep red blood cells/ml. This suspension was then divided into two aliquots and the following were added: (1) an irrelevant antibody, mouse immunoglobulin (IgG) (Sigma) at 10 μg/10^7 sheep red blood cells and (2) monoclonal antibody to sheep red blood cells (mouse IgG anti-sheep red blood cells (Sera Laboratories) at 10 μg/10^7 sheep red blood cells). These mixtures were incubated for 15 minutes at 37°C then washed twice in ice cold PBS. The antibody coated ^{51}Cr sheep red blood cells were then suspended in RPMI–20% heat inactivated fetal calf serum at a concentration of 4×10^7/ml. *C parvum* stimulated mouse peritoneal exudate cells were added to flat-bottomed Removawells (Dynatech) at a concentration of 2×10^5/well in 100 μl of RPMI–10% heat inactivated fetal calf serum. The cells were left to adhere for three hours at 37°C in 5% carbon dioxide, and then washed twice in warm HBSS. The experi-

ment consisted of five separate treatments. To the adherent mouse peritoneal exudate cells 50 μl of one of the following treatments was added: (1) HBSS plus 50 μl of ^{51}Cr labelled sheep red blood cells that had been opsonised in mouse IgG; (2) HBSS alone; (3) *A fumigatus* diffusate; (4) *P ochrochloron* diffusate; (5) a known inhibitor of phagocytosis—cytochalasin B at 25 μg/ml (Sigma). To treatments 2–5 50 μl of ^{51}Cr labelled sheep red blood cells opsonised in mouse IgG anti-sheep red blood cells was added. The Removawells were centrifuged for five minutes at 125 *g*, then incubated at 37°C in 5% carbon dioxide for 1·5 hours. The supernatant was removed and 100 μl of ice cold red cell lytic buffer (to lyse non-phagocytosed sheep red blood cells) was added. After five minutes the lytic buffer was removed and the cells were washed three times in cold PBS. The Removawells containing cells with phagocytosed ^{51}Cr labelled sheep red blood cells were counted in a gamma counter (LKB–Rack Gamma II). For each experiment five replicates were performed.

STATISTICAL ANALYSES

The results of the cell association of the fungal spores were analysed by a three way analysis of variance, the data having been transformed on a logistic scale:

$$\log\left(\frac{\%\text{ cell associated}}{100 - \%\text{ cell associated}}\right).$$

For the sheep red blood cells phagocytosis experiments a two way analysis of variance was used on the log transformed data. Student's *t* tests were used to assess the statistical significance of treatment contrasts, estimates of residual error being obtained from the analyses of variance.

Results

ASSOCIATION OF FUNGAL SPORES AND HUMAN PHAGOCYTIC CELLS

Most (> 77%) of the opsonised spores of both *A fumigatus* and *P ochrochloron* became cell associated with both monocytes and polymorphonuclear cells after incubation in vitro for one hour at 37°C (table 1). No significant differences were found between spore or cell type. Examination by light microscopy of slide preparations of the cell-spore interactions also confirmed that a substantial number of spores had become cell associated. It was not possible, however, to distinguish between attached and ingested cells by means of light microscopy.

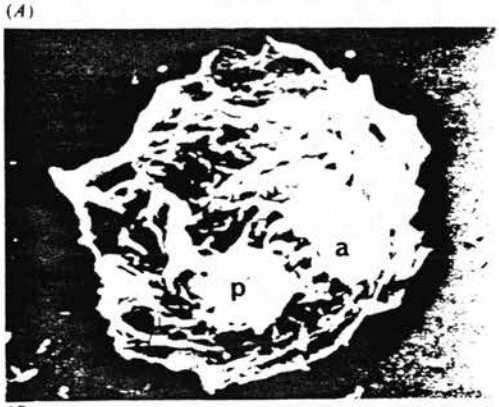
Table 1 *Percentage of opsonised spores that became cell associated after one hour in vitro (means (SEM) of results from the six donors)*

Fungal spores	*Cell type*	*% of cell associated spores*
Aspergillus fumigatus	Monocyte	87·8 (3·2)
A fumigatus	Polymorphonuclear	80·4 (9·5)
Penicillium ochrochloron	Monocyte	80·7 (4·7)
P ochrochloron	Polymorphonuclear	77·5 (6·4)

SCANNING ELECTRON MICROSCOPY OF FUNGAL SPORES AND HUMAN PHAGOCYTIC CELLS

Scanning electron microscopy shows fungal spores as small rounded bodies closely associated with the phagocytic cell membrane. The degree to which the spore has become cell associated has been arbitrarily divided into three phases: (1) attached—spore lying on the cell surface; (2) partially ingested—the cytoplasm of the cell enclosing the spore yet not fully covering it; and (3) ingested—the outline of the spore being seen under the cytoplasm of the cell.

(*A*)



(*B*)

Fig 1 *Scanning electron micrographs of the interaction after 1·5 hours in vitro between spores of Aspergillus fumigatus and (A) a human monocyte and (B) a human polymorphonuclear cell. a—attached spore; p—partially ingested spore.*

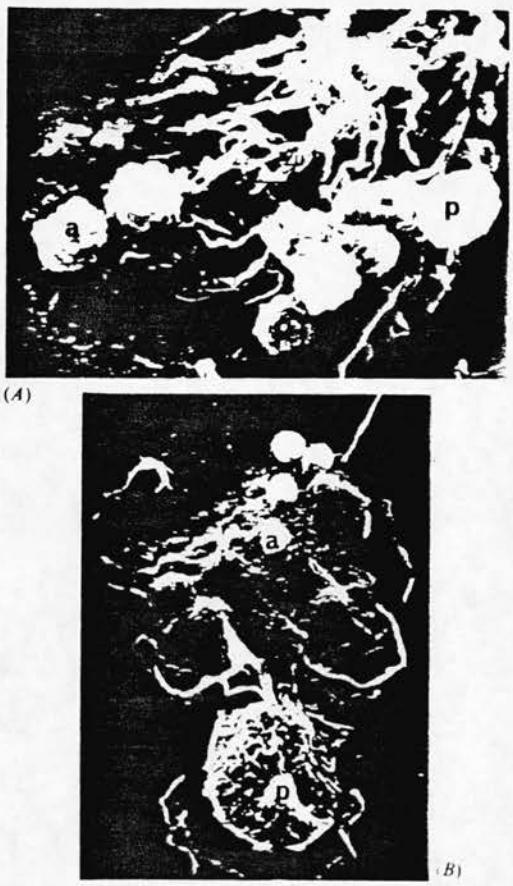
With scanning electron microscopy a substantial number of spores of *A fumigatus* were found to be attached to the surface of the phagocytic cell and not internalised after incubation at 37 C for 1·5 hours (fig 1). Although spores of *P ochrochloron* were present on the surface of the cell they appeared to be less numerous. Opsonisation of the fungal spores in sera containing specific antibody to *A fumigatus* did not appear to alter this finding.

CELL ASSOCIATION OF SPORES WITH MOUSE PHAGOCYTIC CELLS

A mouse model was used to examine whether the finding obtained by in vitro experimentation could be repeated in vivo. We initially examined the interaction in vitro of opsonised fungal spores with thioglycollate elicited mouse peritoneal exudate cells and found that this gave results similar to those obtained with human phagocytes (fig 2). In vivo experiments, in which spores were injected into the peritoneal cavity of the mouse followed with lavage 1·5 hours later, once again gave a similar result. A substantial number of *A fumigatus* spores were seen to be bound to the surface of the cells in vivo, while spores of *P ochrochloron* were in general becoming ingested (fig 3).

PHAGOCYTOSIS OF ANTIBODY COATED ^{51}Cr LABELLED SHEEP RED BLOOD CELLS BY MOUSE PHAGOCYTES

We measured the effect of spore diffusates of *A fumigatus* and *P ochrochloron* on the phagocytosis by *C parvum* stimulated mouse peritoneal exudate cells of antibody coated ^{51}Cr labelled sheep red blood cells. This technique has the advantage that all extracellular (non-ingested) sheep red blood cells, including those attached to the cell surface, may be removed by lysis with red blood cell lytic buffer, thereby enabling an accurate measurement of phagocytosis to be made. There was significantly more phagocytosis ($p < 0·0005$) of the specific anti-sheep red blood cells coated cells than of those coated with irrelevant mouse IgG (table 2). This result established the validity of the technique. The ^{51}Cr labelled sheep red blood cells coated in specific anti-sheep red blood cells antibody were used to assess the effects of the spore diffusate. Diffusates of *A fumigatus* inhibited phagocytosis by about 63% ($p < 0·0025$) while spore diffusates of *P ochrochloron* had no significant effect (table 2). As a positive control we included in the assay 1·25 μg of cytochalasin B, a known inhibitor of phagocytosis; this inhibited the phagocytosis of specific anti-sheep red blood cells coated labelled sheep red blood cells by 44% ($p < 0·005$).



(A) (B)

Fig 2 *Scanning electron micrographs of the interaction in vitro for 1·5 hours of mouse peritoneal exudate cells and (A) spores of Asperigillus fumigatus and (B) spores of Penicillium ochrochloron. a—attached spore, p—partially ingested spore; i—fully ingested spore.*

Discussion

An intriguing aspect of the biological behaviour of *A fumigatus* is its ability to colonise the human bronchopulmonary system under certain circumstances. This has allowed it to become an important pathogen in immunosuppressed patients,[2] a cause of severe postinfluenzal lung infection,[19] and a coloniser of lung cavities,[20] as well as provoking asthma and allergic bronchopulmonary aspergillosis in some people. This association with such a range of diseases makes it unique among fungi pathogenic to man.

Some years ago it was found that *A fumigatus* could be isolated from postmortem human lung more frequently than would have been anticipated from the

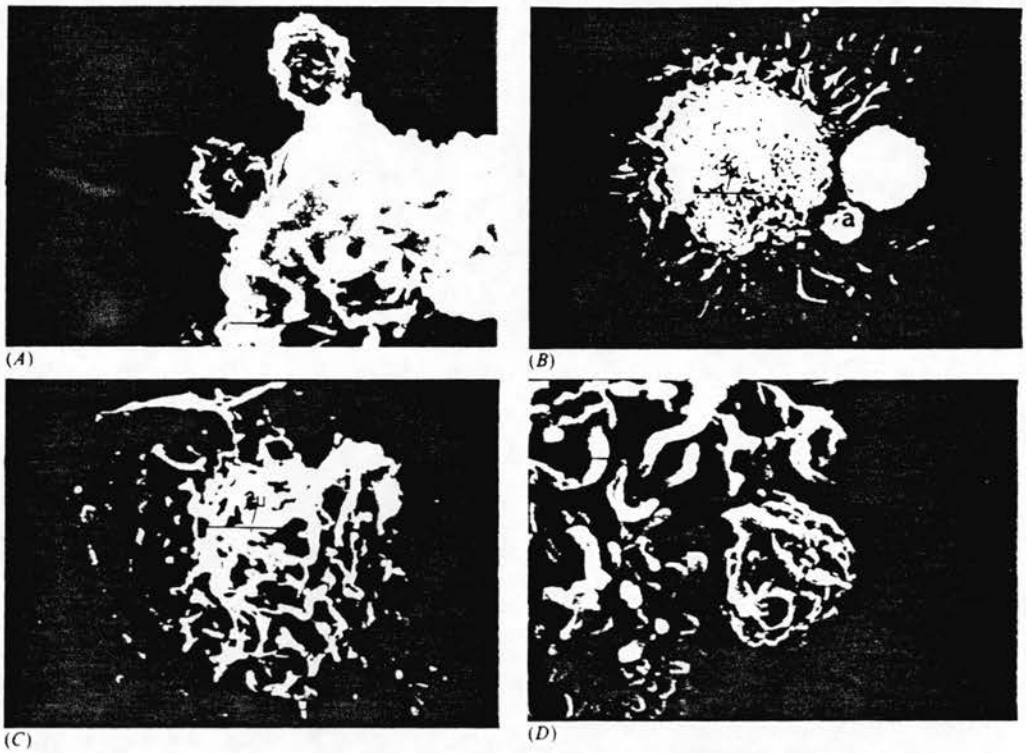


Fig 3 *Scanning electron micrographs of the interaction in vivo for 1·5 hours of mouse peritoneal exudate cells and (A and B) spores of Aspergillus fumigatus and (C and D) spores of Penicillium ochrochloron. a—attached spore, p—partially ingested spore, i—fully ingested spore.*

prevalence of its spores in the air.[21] This led to the suggestion that it might have a specific ability to resist the natural defences of the lung; its small spore size and optimal temperature for germination (37°C) were other factors considered likely to be important. This led us to study the interactions of spores with phagocytic cells in vitro. In a recent publication[13] we showed that spores of *A fumigatus* produce a diffusate containing a low molecular weight factor that inhibits the respiratory burst of neutrophils and macrophages. This factor is given off by the spores after only brief incubation, in contrast to another inhibitory substance, named gliotoxin, shown by others to inhibit phagocytosis by mouse peritoneal exudate cells.[22] Since gliotoxin is produced only after three days' incubation of *A fumigatus*, by which time mycelial growth would be profuse, it is probably a mycelial rather than a spore derived product.

Table 2 *Effect of spore diffusates on the phagocytosis of antibody coated radiolabelled sheep red blood cells (^{51}Cr srbc) by mouse phagocytes (means (SEM) of results of three separate experiments)*

Treatment	*Antibody coating of ^{51}Cr srbc*	*Phagocytosis (CPM)*
HBSS	Mouse IgG	87·1 (10·04)
HBSS	Anti-srbc	440·5 (36·6)
Aspergillus fumigatus diffusate	Anti-srbc	161·8 (29·4)
Penicillium ochrochloron diffusate	Anti-srbc	408·3 (51·6)
Cytochalasin B	Anti-srbc	247·8 (34·2)

HBSS—Hanks' balanced salt solution.

Our finding that *A fumigatus* spore products inhibited the respiratory burst of phagocytic cells led us to consider another important leucocyte function, the process of phagocytosis. In this paper we have shown that the spores of *A fumigatus* are relatively resistant to phagocytosis by human and mouse phagocytes. Initial studies, using light microscopy, did not allow differentiation of ingested and surface bound spores. Scanning electron microscopy did

show that a high proportion of spores of *A fumigatus* (and a lower proportion of spores of *P ochrochloron*) were not taken into the cell but remained bound to the surface. This remained true when cells first encounterd spores in vivo in mice. Attempts were made to quantify this, by means of enzyme stripping techniques, but it proved very difficult to remove spores from cell surfaces. Further efforts, using transmission electron microscopy, also failed as it proved impossible to differentiate between ingested spores and those sectioned between folds of the phagocyte surface membrane. In view of these difficulties in quantifying the effect, we used a reliable assay of phagocytosis, the ingestion of radiolabelled sheep red blood cells coated with antibody. This allowed lysis of any surface associated red blood cells and assay of the numbers actually ingested. Diffusates of *A fumigatus*, unlike those of *P ochrochloron*, significantly inhibited phagocytosis, thus confirming our microscopical observations. This may be the explanation of the findings of Lehrer and Jan,[23] who showed that apparently "phagocytosed" spores (as judged by light microscopy) were nevertheless resistant to killing.

There is thus convincing evidence that *A fumigatus* spores produce one or more substances that readily diffuse from the spore surface and have an inhibitory effect on phagocytosis. Similar mechanisms by which potential pathogens may evade phagocytosis have been shown to be important to the pathogenicity of other microorganisms.[24,25] It has been suggested that evasion of phagocytosis may be a principal method by which microorganisms escape macrophage defence function.[26] The capacity of *A fumigatus* to resist phagocytosis may be an important factor contributing to its pathogenicity, and further studies are now taking place to characterise the diffusate and to examine other aspects of its effects on phagocytic cell function.

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INHIBITION OF PHAGOCYTE MIGRATION AND SPREADING BY SPORE DIFFUSATES OF ASPERGILLUS FUMIGATUS

Maura D. Robertson, A. Seaton, J.A. Raeburn* and L.J.R. Milne*

Institute of Occupational Medicine, Edinburgh
*Western General Hospital, Edinburgh

Address for reprint requests:

Mrs. M.D. Robertson,
Institute of Occupational Medicine,
8 Roxburgh Place,
EDINBURGH EH8 9SU.

ABSTRACT

Previous studies have shown that spores of *Aspergillus fumigatus* inhibit phagocytosis and killing by macrophages and polymorphonuclear leucocytes. In order to identify the mechanisms of this interference with host defences, we have examined the effects of *A. fumigatus* spore diffusates on phagocytic cell function. For comparison, we have used spore diffusates of the non-pathogenic fungus *Penicillium ochrochloron*. The diffusates of *A. fumigatus* reduced the number of human polymorphonuclear leukocytes migrating towards a known chemoattractant by approximately 50% ($p<0.001$). In addition spore diffusates of *A. fumigatus* significantly decreased ($p<0.001$) the capacity of primed mouse peritoneal exudate cells to spread on glass. Spore diffusates of *P. ochrochloron* showed no comparable inhibitory effects. These studies have shown that spore diffusates of *A. fumigatus* inhibit the movement of the phagocytic cell membrane and are thus able to interfere with a primary function of phagocytic cells.

INTRODUCTION

Inhalation of spores of the opportunistic fungus Aspergillus fumigatus may cause allergic disease in man (17) and may in the immunosuppressed person lead to life-threatening lung infection (29). The mechanisms by which this fungus persists in the lung and causes disease in certain individuals is still unclear. It has been suggested that spores of A. fumigatus may be particularly resistant to the actions of the host defence network (20). Phagocytic cells are a primary component of this network, playing an essential role in the eradication of microorganisms (11,14). Previous studies have shown that spores of A. fumigatus are relatively resistant to the actions of phagocytic cells (15,16,25). In addition, we have shown that spores of A. fumigatus release a substance which interferes with important microbicidal functions of phagocytic cells (23,24). As the microbicidal capacity of phagocytic cells is related to their state of functional activation (1,21), we have, in this study, looked at the effect of spore diffusates from A. fumigatus on two aspects of phagocytic cell activation: chemotaxis and spreading. For comparison, we have used spore diffusates of the non-pathogenic fungus Penicillium ochrochloron.

MATERIAL AND METHODS

Phagocytic cells

Human polymorphonuclear leukocytes (PMN) were isolated from the peripheral blood of three healthy donors by density gradient centrifugation (9). The cell populations used comprised >96% PMN with a >95% viability by trypan blue exclusion.

Peritoneal exudate cells (PEC) were harvested from twelve-week old syngeneic C57Bl/6 mice by lavaging the peritoneal cavity with isotonic saline. The mice were pretreated with intraperitoneal injection of Corynebacterium parvum (1.4mg, Wellcome Biotechnology Ltd.), seven days prior to harvesting. This procedure has been shown to increase the ability of phagocytic cells to spread on glass.[8]

Fungal spores and diffusates

The strain of A. fumigatus was an isolate obtained from the sputum of a patient with allergic bronchopulmonary aspergillosis. The strain of P. ochrochloron was IMI 61271. Spores of A. fumigatus and P. ochrochloron were obtained from cultures which had been grown for seven days at 29°C on malt agar. Spore suspensions containing only single, unclumped spores were prepared by gentle homogenisation in Hanks' balanced salt solution (HBSS) followed by filtration through nylon mesh to remove mycelial fragments and chains of spores. Spore counts were performed using improved Neubauer chambers. To obtain diffusates, spores were incubated at a concentration of 10^8/ml in HBSS at 37°C for up to three hours. No visible evidence of germination was present at this time. The spores were removed from the HBSS by centrifugation and the soluble diffusates in the supernatant were passed through 0.22μm sterile filters.

Chemotaxis

In the chemotaxis experiments the basic procedure described by Snyderman (26) for blindwell chambers was followed except that PMN were used. The chemoattractant used was zymosan activated serum which results in the generation of the potent leucotactic factor C5a. Zymosan activated serum (ZAS) was prepared by incubating zymosan (Sigma) with pooled human serum (1mg/ml) for 30 minutes at 37°C. The zymosan was removed from the serum by centrifuging at 500g for 15 minutes and the serum then heated at 56°C for 30 minutes. Two hundred microlitres of the chemoattractant (5% ZAS in RPMI-1640), were placed in the lower compartment which was separated from the top compartment by a 3μm millipore filter. Prior to adding the cells (200μl of $3x10^6$ human PMN per ml of RPMI-20mm Hepes-2% bovine serum albumin) to the top compartment, the following solutions (200μl) were added (i) HBSS, or (ii) A. fumgatus diffusate, or (iii) P. ochrochloron diffusate. For the experiments on random migration, additional treatments were included, in order to determine the chemoattractant properties of the spore diffusates. Instead of ZAS, 200μl of HBSS or 200μl of A. fumigatus diffusate was added to the lower compartment followed by 200μl of HBSS or 200μl of A. fumigatus diffusate to the top compartment. The chambers were incubated in a humidified incubator containing 5% CO_2 for 45 minutes at 37°C by which time a reasonable number of cells have migrated. The filters were then washed and stained and the number of migrated cells, per five high power fields (magnification x 1000), were counted. All tests were carried out in duplicate.

Macrophage spreading assay

Phagocytic cells, stimulated in vivo with C. parvum were used as indicator cells, as they have been shown to demonstrate increased spreading on glass. Fifty microlitres of the following solutions were added to 6 x 22mm glass coverslips: (a) control HBSS, or (b) A. fumigatus diffusate, or (c) P. ochrochloron diffusate. To each of these coverslips was added 50μl of $1x10^5$ C. parvum stimulated mouse PEC contained in RPMI-1640 with 20% fetal calf serum which had been

heat inactivated at 56°C for 30 minutes. The coverslips were then treated according to the method of Donaldson et al (8). This involved incubating the treated coverslips for 1 hour at 37°C. The cells in the coverslips were then washed in saline, fixed in methanol and stained by Giemsa's stain. The coverslips were then mounted, cell side down, onto glass microscope slides. Using a microscope attached to a microcomputer-assisted digitising system, the greatest diameters of 200 cells were measured. All tests were carried out in triplicate.

Cell viability

Trypan blue exclusion was used to assess the viability of the cells following incubation with the spore diffusates under the experimental conditions.

Statistical analysis

The results were analysed using a two-way analysis of variance. Student's 't'-tests were used to assess the statistical significanceof differences in treatment means.

RESULTS

Effect of spore diffusates on chemotaxis of PMN

Spore diffusates of A. fumigatus inhibited the number of cells migrating towards the chemoattractant ZAS by approximately 46% ($p<0.001$). Spore diffusates of P. ochrochloron had no inhibitory effect on the migration of the cells (Table 1). Further experiments which involved measuring the random migration of the cells in HBSS alone versus A. fumigatus diffusates in the lower compartment (Table 2) confirmed that the spore diffusates of A. fumigatus were not acting as a chemoattractant. A comparison of the effect of eight different preparations of A. fumigatus diffusate from isolates of the same strain on the chemotaxis of human PMN towards the known chemoattractant, ZAS, showed that this inhibitory activity was reproducible ($p<0.001$) (Table 3).

Effect of spore diffusates on phagocytic cell spreading

Spore diffusates of A. fumigatus were seen to inhibit the spreading of C. parvum stimulated mouse PEC when compared with control HBSS and with spore diffusates of P. ochrochloron (see Figure). Measurement of the mean diameters of the cells showed that those cells treated with A. fumigatus diffusate were substantially smaller, by approximately 54%, than those with control HBSS (Table 4). The differences between cells treated with A. fumigatus diffusate and the controls were significant (both $p<0.001$).

Effect of spore diffusates on cell viability

The viability of the phagocytic cells incubated with the spore diffusate was the same as that found after incubation in HBSS (>95% viable) indicating that the spore diffusate was not cytotoxic. The pH of the diffusate was 7.1 (the same as the HBSS).

DISCUSSION

When phagocytic cells come into contact with microorganisms they respond by undergoing a series of reactions which are collectively referred to as activation (7). This process may include phagocytosis (27), the release of reactive oxygen intermediates (3), and secretion of other soluble mediators including substances which attract other cells to the active site (10,31). Previous studies in our laboratories have shown that spores of A. fumigatus are relatively resistant to phagocytosis (22) and that they fail to trigger the release, by phagocytic cells, of the potentially microbicidal reactive oxygen intermediates (23). Moreover, spore diffusates of A. fumigatus inhibit the phagocytosis of antibody-coated, radiolabelled sheep red blood cells and suppress the spontaneous release of reactive oxygen intermediates by C. parvum-stimulated mouse phagocytic cells (23,24). Thus it appears that A. fumigatus spores release a diffusable substance that has several effects of considerable biological importance in ensuring their survival in the lung and therefore enhancing their pathogenic potential. Preliminary investigations have already shown that the spore diffusate is released immediately spores are place in aqueous solution. This suggests that it is a constituent present on or close to the surface of the spore rather than a metabolite which is produced as a result of germination. The inhibitory factor is dialysable and so has a molecular weight of less than 14,000 daltons (23). It has a dose-dependent effect on the inhibition of reactive oxygen intermediates by phagocytic cells (23). The precise biochemical nature of this soluble diffusate is currently under investigation. We have now attempted to pinpoint the effector mechanism of the spore diffusate by examining its effect on cell movement - a fundamental aspect of phagocytic cell activation. In order for a phagocytic cell to become activated thereby enhancing its phagocytic ability, a rearrangement of the cell membrane must occur (12,32). This involves a complex process which requires a coordinated interaction of the plasma membrane with contractile elements in the cytoplasm (13,28). The changes are reflected in an

enhanced ability of the cell to spread over a surface and to migrate in response to chemical stimuli. Such functions are readily measured *in vitro*, and we have been able to show that both are inhibited by the diffusate of *A. fumigatus* spores. In contrast, spore diffusates of *P. ochrochloron* had no effect. Futher work confirmed that the spore diffusate of *A. fumigatus* was not acting as a chemoattractant.

It therefore seems likely that spores of *A. fumigatus* are able to resist attack by phagocytes by interfering with a fundamental aspect of phagocytic cell activation thereby having both an inhibitory effect on phagocytosis and by affecting the movement of other accessory cells which may be recruited to the site as part of the host defence antimicrobial network (4). Our previous work has shown, however, that spores do become attached to the surface of phagocytes, both *in vivo* and *in vitro* (22,23) and it is probable that the inhibitory effects are only partially effective in preventing removal and killing of the spores. Indeed it is clear from clinical experience that phagocytes play an important part in preventing invasive aspergillosis in that this disease occurs particularly in patients with acute leukaemia and chronic granulomatous disease (2,6). The results of other *in vitro* studies have shown that the supernatants of 3-day old cultures of *A. fumigatus* possess substances which interfere with the functions of immunocompetent cells (5,19), whilst 7-day old cultures interfere with the opsonic properties of complement (30). The important difference between our results and those of other workers (5,19,30) is that the inhibitory effect is produced by a substance obtained from freshly released spores which are the elements that first encounter the host defences.

Several questions remain to be answered with respect to the pathogenicity of *A. fumigatus*. What is the precise nature of the diffusate and how does it affect phagocytic cell membranes? As *A. fumigatus* is so successful in resisting host defences, why is it not more frequently a cause of lung disease since its spores are inhaled by all individuals throughout the year? Why is it able to

thrive in asthmatic, as opposed to bronchitic, airways? What are the effects of corticosteroids in predisposing patients to develop invasive disease? In view of the interactions between macrophages and lymphocytes in normal host defence and the occasional occurrence of invasive aspergillosis in patients after influenza (18), what role do lymphocytes play in the elimination of the organism? The answers to these questions may have implications for future management of opportunistic infections and allergic bronchopulmonary aspergillosis.

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TABLE 1

The effect of spore diffusates on the migration of human PMN towards zymosan activated serum

Experiment	Treatment		
	HBSS* control	*A. fumigatus* diffusate	*P. ochrochloron* diffusate
1	68.5 (4.8)	32.6 (3.4)	68.4 (6.0)
2	62.9 (1.2)	36.2 (4.8)	62.6 (0.0)
3	83.3 (7.6)	46.2 (0.0)	ND

Results of three separate experiments expressed as the mean (SD) of the number of cells migrated per 5 high power fields

*Hanks' balanced salt solution

ND = not done

TABLE 2

Effect of A. fumigatus diffusates on random migration of human PMN cells

Treatment		No. of cells migrated per 5 high power fields
Top compartment	Lower compartment	
HBSS*	HBSS	3.0 (1.01)
A. fumigatus diffusate	A. fumigatus diffusate	1.53 (0.39)
HBSS	A. fumigatus diffusate	2.73 (0.43)

Results expressed as the mean (SEM) of two experiments

*Hanks' balanced salt solution

TABLE 3

The effect of 8 different preparations of A. fumigatus diffusate on the migration of human PMN towards zymosan activated serum

A. fumigatus diffusate preparation	% of inhibition of migration
1	52.7
2	44.0
3	41.8
4	50.3
5	56.6
6	44.5
7	49.0
8	50.9

TABLE 4

The effect of spore diffusates on the spreading of *C. parvum*-stimulated mouse peritoneal exudate cells

Treatment	Cell diameter Mean (SD)	Percentage inhibition of spreading
*HBSS control	20.2 (2.9)	-
A. fumigatus diffusate	9.2 (0.5)	45.5
P. ochrochloron diffusate	19.3 (2.6)	4.5

Results given are the mean (SD) of three separate experiments

*Hanks' balanced salt solution

CAPTION FOR FIGURE

The effect of spore diffusates on the spreading of *C. parvum*-stimulated mouse peritoneal exudate cells.

(a) Hanks' balanced salt solution (control)
(b) *A. fumigatus* diffusate
(c) *P. ochrochloron* diffusate


(a)
(b)
(c)

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